1-1-2018

Identifying Molecular Mechanisms of Immunomodulation by Staphylococcal Superantigens in Humans

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Identifying molecular mechanisms of immunomodulation by staphylococcal superantigens in humans

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2018

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Superantigens are exotoxins produced by *Staphylococcus aureus* and induce extensive T cell proliferation and proinflammatory cytokines, leading to toxic shock syndrome at high concentrations. However, the role of superantigens produced at relatively low concentrations during asymptomatic colonization or chronic infection has not been well established. In this dissertation, we demonstrated that stimulation of human PBMCs with staphylococcal enterotoxin C1 (SEC1) at the dose inducing a half maximal T cell proliferation (suboptimal stimulation) induced immunosuppressive CD4+CD25+FOXP3+ and CD8+CD25+FOXP3+ T cells. The suppression of these cells was mainly mediated by the galectin-1. We found that suboptimal stimulation with SEC1 induced differential activation of PI3K-mTOR-Akt pathway, leading to expression of FOXP3 isoforms preferably localized to the nucleus and induction of PTEN that contributes to maintain stability and suppressive activity of regulatory T cells. Taken together, these results demonstrate the important role of superantigen produced at low concentration during asymptomatic colonization that induce immunosuppressive CD4+

and CD8+ regulatory T cells to promote survival, propagation, and colonization for *S. aureus* in the host.

DEDICATION

I would like to dedicate this research to my family.

ACKNOWLEDGEMENTS

I am grateful to all of those with whom I have had the pleasure to work during this and other related projects. Firstly, I would like to express my sincere gratitude to my advisor, Dr. Keun Seok Seo for the continuous support of my Ph.D. study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this dissertation. I would like to thank my dissertation committee, Dr. Stephen Pruett, Dr. Lesya Pinchuk, and Dr. Barbara Kaplan, for their insightful comments and encouragement, but also for the hard questions which incented me to widen my research from various perspectives.

I am thankful to my fellow lab mates for their feedback and cooperation, and for all the fun we had during these past years. I owe great thanks to Dr. Nogi Park for his enthusiasm about the research and willingness to help whenever I ask. I was fortunate to work with him as a teammate for this project. I also would like to thank all the blood donors who volunteered for this project and the nurses who helped us to collect blood samples.

I would like to thank my fellow doctoral students for their friendship, support, and unforgettable memories. In addition, I would like to express my gratitude to John Stokes and Dr. Wei Tan from the Flow Cytometry Core Facility at college of veterinary

medicine, for their assistance and the last minutes favors. My gratitude extends to Dr. Juw Won Park at University of Louisville for the incredible RNA-seq data analysis.

This project would not have been possible without support by the Center for Biomedical Research Excellence in Pathogen–Host Interactions, National Institute of General Medical Sciences, National Institutes of Health.

Last but not the least, I would like to thank my dad, mom, sister and brother-in-law and my best friend, Mi-young Kwak, for their boundless support and for believing in me.

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CHAPTER I

INTRODUCTION AND REVIEW OF RELAVANT LITERATURE

1.1 Introduction

Staphylococcus aureus (*S. aureus*) is a gram-positive coccus frequently colonizing skin, nasal mucosa, and respiratory tract of humans. It causes a multitude of diseases from mild soft tissue infections to life threating diseases such as infective endocarditis, sepsis, necrotizing pneumonia, and toxic shock syndrome (TSS) [1]. Approximately 30-40 % of people are colonized asymptomatically at any given time and 70 % of people experience *S. aureus* infection without any symptoms in their lifetime [1]. Pathogenesis of *S. aureus* infection is mediated by various virulence factors, among which the staphylococcal superantigens play a critical role on host immune responses.

Superantigens (SAgs) are classically known to modulate host immune responses by their properties to activate enormous number of T cells, leading to cytokine storms and anergy following activation-induced cell death [1]. The pathologic role of SAgs is well characterized in toxic shock syndrome (TSS) at which a large amount of SAgs ranging from 1 to 10 μg/ml were produced [2]. However, in chronic infections or asymptomatic colonization, *S. aureus* produces a small quantity of superantigens that hardly induce detectible level of serum antibodies [3-5] and the role of such small amount of SAgs in the *S. aureus* pathogenesis has not been clearly demonstrated yet. Furthermore, several population genomic studies demonstrated that the most prevalent

SAg genes in *S. aureus* clinical isolates are the enterotoxin gene cluster SAgs (SEG, SEI, SElM, SElN, SElO) of which expression is intrinsically regulated to at low amounts [6].

Regulatory T cells (Tregs) play an important role in maintaining immune homeostasis and preventing autoimmune diseases [7-10]. However, pathogens can exploit Tregs to establish infection by suppressing host immune response. Importantly, suboptimal stimulation of T cell induce development of Treg in thymus and periphery. These suggest that a low amount of SAgs produced by *S. aureus* might induce Treg to promote its colonization and pathogenesis which have not been clearly demonstrated yet.

1.2 Regulatory T cells

1.2.1 Brief history

The presence of immunoregulatory mechanisms that control peripheral immune tolerance against self-reactive immune cells had been suggested before discovery of Tregs. Studies in 1970s showed that thymus-derived lymphocytes seemed to contribute to control of autoimmune diseases, as thymectomized mice and rats developed severe autoimmune diseases, which could be prevented by transfer of T cells from normal mice or rats [11-13]. However, efforts to identify the suppressive T cells failed due to difficulties in discovering specific marker and suppression mechanisms, and even in preparation of suppressive T cells until identification of CD25 (IL-2 receptor α chain) as a specific marker of the suppressive CD4+ T cells by Sakaguchi et al in 1995 [14]. The role of CD4+CD25+ T cells on maintaining immune tolerance was clearly demonstrated in mice, followed by identification of counterpart CD4+CD25+ T cells in humans in 2001 [15, 16].

To identify molecular mechanisms of Treg development, researchers had focused on genetic defects in recessive inflammatory diseases observed in scurfy mice [17] and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans [18]. Genetic analysis revealed that a single gene mutation in a transcription factor, forkhead box P3 (Foxp3), in scurfy mice rapidly developed fatal autoimmune diseases [19]. Immediately, mutations of human FOXP3 in IPEX patients were identified [18, 20], which suggested possible role of Foxp3 in immunoregulation. Subsequent studies determined that Foxp3 was undoubtedly a key transcription factor for CD4+CD25+ Tregs. High expression of Foxp3 mRNA was detected in CD4+CD25+ T cells and ectopic expression of Foxp3 conferred Treg-like phenotype and functionality on peripheral CD4+CD25- T cells [21, 22]. Eventually, the suppressive CD4+ T cells were defined as CD4+CD25+FOXP3+ regulatory T cells.

Subsets of CD8+ T cells suppressing immune responses have been introduced in 1970 [23], but CD8+ Tregs had been left behind until discovery of CD4+ Tregs due to high heterogeneity in cell phenotypes and lack of knowledge about the suppression mechanism. Still, the phenotypic characteristics of CD8+ Tregs are much broader depending on the induction methods and no unique marker has been described yet, even Foxp3. Although lack of CD28 expression has been suggested as a marker for CD8+ Tregs [24], CD8+CD28+ T cells with suppressive activity are present in mice and humans [8, 25]. Knowledge on CD8+ Tregs is growing, especially as it relates to clinical significance in autoimmune diseases and cancer.

1.2.2 Subsets of regulatory T cells

Following the current nomenclature recommendation [11], Tregs can be classified based on the anatomical origin of cells. Tregs are primarily generated from thymus (thymus-derived Tregs, tTregs) that survived thymic selection. Tregs also can be induced from naïve T cells by stimulation in periphery (pTregs). Tregs induced by *in vitro* stimulation of naïve T cells are termed in vitro-induced Tregs (iTregs) to distinguish them from pTregs. It remains unclear whether pTregs and iTregs represent identical subpopulations. The presence of TGF-β is essential to induce iTregs in mice whereas human iTregs can be induced without addition of TGF-β. It is suggested that suppression mechanisms employed by tTregs and iTregs may differ [12]. tTregs appear to play a primary role in preventing differentiation of T cells into pathogenic Th1 cells and regulating effector T cell trafficking *in vivo* [13, 14]. Meanwhile, iTregs modulate DC function by producing IL-10 and downregulating costimulatory molecules, thereby inhibiting effector T cell functions [15]. Antigen-specific iTregs seem more effective to prevent and treat organ specific autoimmunity than tTregs in mouse model study [16].

1.2.3 FOXP3

FOXP3 is a transcription factor indispensable for Treg development and function. Mutations in *FOXP3* gene result in deficiency in functional Tregs and severe multi-organ immune disorder such as scurfy syndrome in mice [26] and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans [27], indicating an essential role of FOXP3 in Treg function. The *FOXP3* gene consists of 12 exons, but the first exon is not translated. Hence, *FOXP3* gene is generally considered to have 11 exons that encodes a protein consisting of several functional domains including a

N-terminal domain, a C2H2 zinc finger, a leucine zipper, and a winged-helix/forkhead DNA binding domain. FOXP3 isoforms by alternative splicing have been identified in human Tregs while only full-length protein is reported in the mouse counterpart. Thus far, FOXP3 isoforms lacking exon 2 (ΔE2), exon 7 (ΔE7), and exon 2 and exon 7 (ΔE2ΔE7) have been reported [28-30]. Exon 2 is a part of proline-rich region at N-terminal domain of FOXP3 that interact with RORα and RORγt. Exon 7 is part of leucine zipper domain necessary for FOXP3 homodimerization. ΔE2ΔE7 does not affect dimerization but abrogate suppressive activity [28, 31]. The function of FOXP3ΔE7 remains unknown [30].

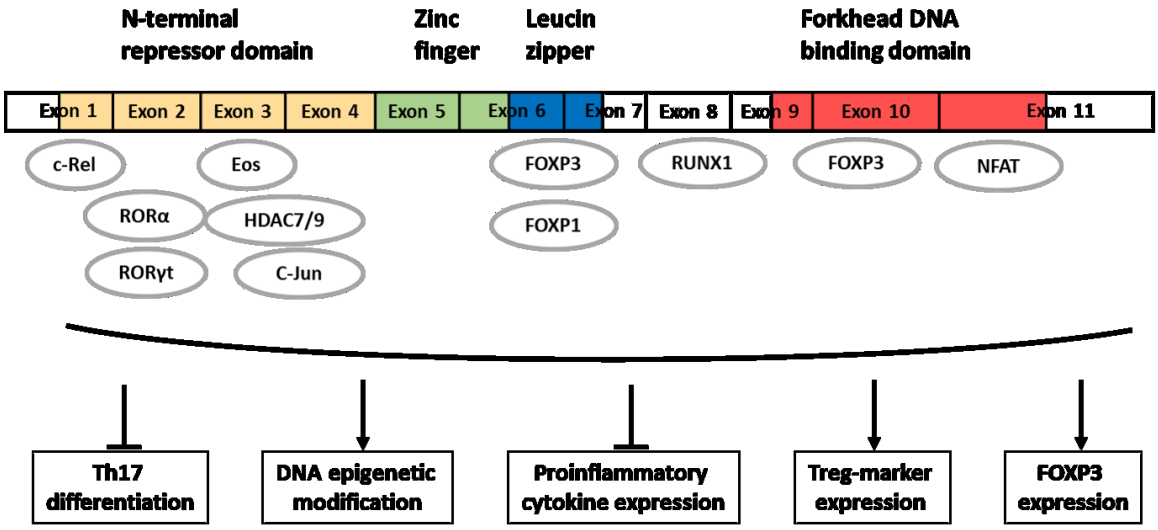

Figure 1.1 FOXP3 structure and its binding partners.

ROR: RAR-related orphan receptor, HDAC: Histone deacetylase, RUNX1: Runt-related transcription factor 1, NFAT: Nuclear factor of activated T cells

FOXP3 binds to more than 2000 genes differentially expressed in Tregs and suppresses or promotes expression of target genes [32, 33]. Proteomic study further revealed that FOXP3 forms a large functional complex of ~600 kDa with more than 300

proteins including transcriptional factors [34]. These interactions are implicated in various biological processes such as DNA binding, transcription regulation, and chromatin modification [35, 36]. (Figure 1.1). For example, the N-terminus of FOXP3 interacts with c-Jun and inhibits its nuclear localization, subsequently downregulating cell activation process and maintaining unresponsiveness [37]. The exon 1-encoding region located in N-terminal domain of FOXP3 interacts with c-Rel, a member of NF-κB, and suppress gene expression of proinflammatory cytokines such as IL-2, IL-12, and IFN-γ [38]. Also, the exon 2-encoding region, lacking in the ΔE2 isoform, is shown to antagonize Th17 development by interacting RORα and RORγt [39]. When NFAT binds to forkhead domain of FOXP3, this represses IL-2 expression and upregulates expression of Treg-phenotypes such as CTLA-4 and CD25 [40].

FOXP3 localization is regulated by multiple factors. Nuclear import sequence is reported as arginine-lysine-lysine-arginine (RKKR) sequence located in forkhead domain [41, 42]. It is also suggested that FOXP3 translocation may be achieved by interacting with other transcriptional factors and co-transporting with them [43]. Nuclear export signal sequences are predicted to be present in boundary zone of exon 1 and 2, and exon 6 and 7, respectively. Thus, FOXP3 ΔE2 is preferably localized to the nucleus, while full length FOXP3 is preferably localized to cytoplasm [42]. Several studies demonstrated expression of FOXP3 ΔE2 in tTreg and iTreg are higher than other Foxp3 isoforms, suggesting an important role of FOXP3 ΔE2 localized in the nucleus for functional maturation of Treg [28, 44]. However, the role of FOXP3 ΔE2 is still controversial. Transduction of ΔE2 using retroviral vector in primary CD4+CD25- T cells resulted in

suppressive activity [28, 45] or not [29]. These results suggest that expression of FOXP3 is necessary but not sufficient for the functional maturation of Treg.

1.2.4 Induction of FOXP3 expression

Despite extensive research, the underlying mechanisms of Tregs induction remain elusive. During thymic selection, some CD4+CD25+ thymocytes survived negative selection and become CD4+CD25+Foxp3+ tTregs [46]. These CD4+CD25+Foxp3+ thymocytes have moderate to low affinity to self-antigen, suggesting the strength of TCR stimulation modulated by the affinity of TCR to antigen seems to play an important role in Tregs induction [47-49]. Similar to these findings, stimulation of murine CD4+ T cells with a low dose of specific or non-specific antigen to TCR in the presence of dendritic cells induced immunosuppressive CD4+CD25+ Foxp3+ Tregs, while a high dose stimulation induced CD4+CD25+ effector T cells [47]. In humans, a low dose antigen promotes induction and proliferation of antigen-specific FOXP3+ Tregs whereas a high dose antigen favors to generate FOXP3- effector T cells [50].

The signaling downstream of TCR via PI3K, p110α, p110δ, Akt, and mTOR play an important role in expression of Foxp3 in tTreg and iTreg. Inhibition of these signaling pathways induced expression of Foxp3 and Treg-like mRNA and miRNA profiles [51]. On the contrary, constitutive activation of the PI3K/AKT/mTOR pathways in PTEN deficient T cells significantly reduced Foxp3 expression [52]. The role of co-stimulatory signal through CD28 is controversial. As the CD28-deficient mice have ~80% reduced thymic CD4+CD25+Foxp3+ Tregs, costimulatory signals via CD28 seems to be important for induction of tTregs [53]. In contrast, strong activation of CD28 via anti-CD28 antibody inhibits induction of Foxp3. Indeed, activation of TCR and CD28 by a

low dose of antigen and anti-CD28, followed by TCR signal deprivation and PI3K/mTOR inhibition induced higher expression of Foxp3 [51]. These results suggest that expression of Foxp3 is regulated by the timing of PI3K/AKT/mTOR pathways inhibition upon activation of T cell via TCR signaling [51]. Major cytokines involved in Tregs induction are IL-2 and TGF-β [54, 55]. Binding of IL-2 to its receptor induces activation of signal transducer and activator of transcription 5 (STAT5), which binds to the promoter and enhancer region of *FOXP3* [55]. TGF- β1 signaling promotes the binding of nuclear factor of activated T cells (NFAT) and mothers against decapentaplegic homologue 3 (SMAD3) to the enhancer region of *FOXP3* and in turn control epigenetic regulation of Foxp3 [56, 57].

1.2.5 Mechanisms of regulatory T cell-mediated suppression

Diverse suppression mechanisms (Table 1.1 and 1.2) have been proposed for Treg depending on the type of Tregs (tTregs, pTregs, iTregs), induction mechanism (anti-CD3/CD28 beads, antibodies, soluble antigens, and superantigens), and species (human, mouse). The suppression mechanism can be broadly classified into two categories: cell-to-cell contact-dependent and -independent suppression. The cell-to-cell contact-dependent suppression requires direct interactions between Tregs and effector T cells and/or antigen presenting cells (APCs). On the other hand, contact-independent suppression is mediated by soluble factors such as immunosuppressive cytokines/chemokines, cytolytic molecules, and changes in microenvironment necessary for T cell proliferation [58, 59].

Table 1.1 Suppression mechanisms of CD4+ Tregs

Key molecule	Species	Type	Induction	Mechanism	Ref.
INHIBITORY CYTOKINE S AND CHEMOKINES					
CCL3/4	mouse	thymic		Attracts CCR5+ T cells in vivo Inhibits TCR signaling by inhibiting Ca2+ flux in T cells	[60]
TGF-β	human	adaptive	SEB, TGF-β	Inhibit T cell proliferation by repressing gene expression of cytokine receptors and by inducing cbl-b Induce apoptosis via pro-apoptotic bcl-2 family proteins Surface-bound TGF-β complexed with GARP mediates contact-dependent suppression	[61]
	mouse	thymic			[62]
		adaptive	Activated tTregs		[63]
IL-10	human	thymic		Downregulates costimulatory molecules and production of proinflammatory cytokines	[64]
		adaptive	Helicobacter pylori infection		[65]
	mouse	thymic			[66, 67]
		adaptive	Activated tTregs		[63]
IL-35	human	adaptive	IL-35, anti-CD3/anti-CD28	Inhibits T cell proliferation and production of proinflammatory cytokines	[68]
	mouse	thymic			[66, 69]
CYTOLYTIC MOLECULES					
Granzyme B	human	thymic		Induces apoptosis in effector T cells, dendritic cells, and natural killer cells in both a perforin-dependent and -independent manner	[70]
	mouse	thymic			[71]
Galectin-1	human	thymic		Triggers Ca2+ efflux, leading to arrest and apoptosis of activated effector T cells	[72]
Galectin-3	human	thymic		LGALS3 mRNA is highly expressed in human Tregs but the functionality has not been tested yet	[73, 74]
Galectin-9	mouse	thymic		Binds to DR3 (TNFRSF25) expressed on Tregs and required for Treg function	[75]
		adaptive	Concanavalin A injection to induce hepatitis		[76]
Galectin-10	human	thymic		Treatment of galectin-10-specific siRNA results in partial abrogation of Treg function, but its ligand and mechanisms are unknown	[77]
IMMUNOSUPPRESSIVE METABOLITES					
COX2	human	adaptive	SEB, anti-CD3/anti-CD28, immature DC, tumor cells (COX2+/-), IL-2, IL-10, and IL-15	Facilitate production of PGE2 which binds to EP2 and EP4 receptors, inducing upregulation of intracellular cAMP	[78, 79]
CD39/CD73	human	thymic		Cleave ATP sequentially into adenosine which binds to A2A receptors expressed on conventional T cells, leading to upregulation of intracellular cAMP	[80, 81]
	mouse	thymic			[82]
cAMP	mouse	thymic		Downregulates proinflammatory cytokines expression in T cells and APCs and CD80/CD86 expression by DCs Suppression is mediated by transfer of cAMP at the cell-contact junction	[83, 84]

Table 1.1 (Continued)

IDO	mouse	thymic	Tolerogenic DCs induced by tTregs	IDO expression in DCs is induced by tTregs through CTLA-4 dependent mechanism IDO facilitate tryptophan metabolism, which induces production of pro-apoptotic metabolites such as kynurenine	[85]
		adaptive	Repeated *in vivo* injection of SEB		[86]
CONTACT-DEPENDENT SUPPRESSIVE MOLECULES					
CTLA-4	mouse	thymic		Binds to CD80/CD86 on APCs with higher affinity than CD28, inducing downregulation of CD80/CD86. CTLA-4 ligation also induces IDO expression in DCs	[87, 88]
PD-1	mouse	adaptive	LCMV chronic infection	Binds to PD-L1 and induces inhibitory signal, preventing activation and proliferation of CD8+ T cells	[89]
LFA-1 (CD11α/ CD18)	human	thymic		LFA-1 on Tregs interact with ICAM-1 on DCs, inhibiting CD80 and CD86 expressions in DCs	[90]
	mouse	thymic			[91, 92]
LAG-3	mouse	thymic		Binds to MHC class II molecules with a higher affinity than CD4 and induces ITAM-mediated inhibitory signals, thereby downregulating DC function	[93]
neuropilin-1	mouse	thymic		Promote prolonged interactions of Tregs with DCs. Activated human T cells express neuropilin-1	[94-96]
FasL	human	thymic		Binds to Fas receptor and induces cytolysis of effector T cells and DCs	[97, 98]
	mouse	thymic		Alter DC function	[99]
TRAIL	mouse	thymic		Induces T cell death via TRAIL-DR5 pathway	[100]

COX2: Cyclooxygense-2, IDO: Indolamine-2,3-dioxygenase, LCMV: lymphocytic choriomeningitis virus, TRAIL: TNF-related apoptosis-inducing ligand, DR5: Death receptor 5

Table 1.2 Suppression mechanisms of CD8+ Tregs

Key molecule	Species	Type	Induction	Phenotype	Mechanism	Ref.
INHIBITORY CYTOKINES AND CHEMOKINES						
CCL4	human	adaptive	bacillus Calmette-Guerin's stimulation	CD8+CD25+ FOXP3+LAG3+	Inhibit TCR signaling by inhibiting Ca2+ flux in T cells	[25]
IL-10	human	adaptive	allogeneic CD40 ligand-activated DC derived from monocyte	CD8+IL-10+	Downregulates costimulatory molecules and production of proinflammatory cytokines	[101]
	human	adaptive	tumor pDC derived from macrophage	CD8+CCR7+ CD45RO+IL-10+		[102]
	human	adaptive	Monocytes, GM-CSF, IL-2	CD8+CD28-		[103]
	mouse	thymic		CD8+CD122+		[104-106]

Table 1.2 (Continued)

TGF-β	human	thymic		CD8+CD25+	Inhibit T cell proliferation by repressing gene expression of cytokine receptors and inducing cbl-b Induce apoptosis via pro-apoptotic bcl-2 family proteins Surface-bound TGF-β complexed with GARP mediates contact-dependent suppression	[107]
	mouse	thymic		CD8+CD28-		[108]
	mouse	adaptive	antigen specific stimulation	$CD8^+CD62L^+$ $GITR^+TGF\beta^+$		[109]
	mouse	adaptive	TNFΔARE mice	CD8+CD44+ CD103+		[110]
IFN-γ	human	adaptive	autologous irradiated MN, GM-CSF, IL-2	CD8+	In SLE patient, IL-6 also involves in suppression Mediates suppression in *in vivo* EAE model, partially perforin-dependent	[111]
	human	adaptive	autologous irradiated MN and conditioned media	CD8+		[112]
	mouse	adaptive	glatiramer acetate (Copaxone®)	CD8+		[113]
IMMUNOSUPPRESSIVE METABOLITES						
IDO	mouse	adaptive	anti-4-1BB mAb	CD8+CD11c+	Promotes tryptophan catabolism, generating metabolites cytotoxic to T cells	[114]
NOX2	human	adaptive	anti-CD3 mAb (OKT3), IL-15	CD8+CCR7+	Induces ROS in CD4 T cells, inhibiting phosphorylation of ZAP70 and LAT	[115]
CONTACT-DEPENDENT SUPPRESSIVE MOLECULES						
CTLA-4	human	thymic	thymocytes	CD8+CD25+	Alter CD80/86 expression on DCs and maturation of DC	[107]
	human	adaptive	autologous LPS-activated DC	CD8+CD25+ CTLA4+FOXP3+		[116]
ILT-3/ ILT-4	human	adaptive	antigen-pulsed autologous APC	CD8+CD28- FOXP3+	Induce ILT-3 and ILT-4 on APC which induce T cell anergy	[117]
unknown	human	adaptive	SEB	CD8+CD25+ FOXP3+	no suppression in TW	[9]
	human	adaptive	anti-CD3 mAb (hOKT3γ1)	CD8+CD25+	no suppression in TW	[118]
	human	adaptive	SEA, TSST-1, SPE-K/L	CD8+CD25+ FOXP3+	no suppression in TW	[119]
	mouse	adaptive	irradiated THP-1 cell	CD8+CD28- CD56+	Unknown	[120]

pDC: plasmacytoid dendritic cell, NOX2: NADPH oxidase 2, TW: transwell

1.2.6 Regulatory T cells in mice and humans

Mice have been an essential model for characterizing Tregs in vivo. The Tregs in mice and humans share common features including; 1) expression of Foxp3 in

CD4+CD25+ T cells; 2) induction from thymus (tTreg) and periphery (pTreg); 3) suppression by cell-to-cell contact dependent and independent-manner. However, there are some fundamental differences between mice and human Tregs. The most prominent and significant differences are in Foxp3. In mice, Foxp3 is exclusively expressed in tTregs and pTreg which allows Foxp3 as a definite marker for Tregs in mice [20, 121]. By contrast, in humans, FOXP3 can be transiently expressed in CD4+CD25+ T cells without immunosuppressive function [122, 123]. Whereas mice only express a single form of Foxp3 as in full length, humans express various Foxp3 isoforms as described above [30, 44, 124] and it is not clearly demonstrated yet which Foxp3 isoforms are critical for development and maturation of Tregs in humans. Thus, Foxp3 cannot be an exclusive marker for functional Tregs in humans.

The suppression mechanisms are likely to differ between mouse and human Tregs as described in Table 1.1 and 1.2. Not all suppressive molecules found in one species can be applied in the other species. Some suppressive mechanisms such as via CTLA-4 and LAG-3 have only shown in mouse models. In the case of IL-35, mouse tTregs constitutively express IL-35 whereas human Tregs do not express IL-35 unless stimulated with IL-35 provided from other cells [68, 125]. Neuropilin-1 is suggested as a distinct cell surface marker to differentiate tTregs from pTregs in mice [95], but in humans, neuropilin-1 is upregulated in activated T cells [96]. Neuropilin-1 plays a role, not only in maintaining prolonged Tregs-DC interaction [94-96] but in capturing a latent form of TGF-β and convert it to an active form [126], indicating importance of neuropilin-1 on TGF-β-mediated suppression mechanism. Therefore, being aware of differences between

mouse and human Tregs is required to minimize confusion on understanding physiology and functionality of Tregs.

1.2.7 Regulatory T cells in infection

In infectious disease, immune responses to pathogens can result in severe damage to host tissues. Tregs play an essential role in controlling such immunopathological processes. Tregs suppress robust immune responses to infections, thereby promoting microbial persistence that arises via the balance between inflammatory immune responses and immunosuppression by Tregs [127]. While most pathogens have developed multiple strategies to evade from host immune systems, they may favor to utilize Tregs for their survival. In fact, Tregs become fully functional featured by enhanced production of immunomodulatory cytokines such as IL-10 and TGF-β upon activation with microbial antigens [128, 129]. Since TGF-β is essential not only for maintenance of Tregs function but for induction of Tregs from conventional T cells, the microenvironment surrounding activated Tregs in certain microbial infection may provide more favorable condition to Tregs.

The immunomodulatory effect by Tregs has been understood in pathogens that cause chronic infection. Increased Tregs population has been reported in patients chronically infected with *Helicobacter pylori* [65, 130, 131] or *Mycobacterium tuberculosis* [132]. Children with nasopharyngeal carriage of *Streptococcus pneumococcus* had higher portion of Tregs in adenoidal tissues [133]. In addition to bacterial infections, increased population of CD4+ and CD8+ Tregs have been reported in chronic and acute viral infections [134-138], parasite chronic infections [139-141], and fungal infections [142, 143].

1.3 Staphylococcal superantigens

1.3.1 Classification and structures

Staphylococcal superantigens are a family of at least 24 serologically distinct proteins including toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEA to SEE), and SE-related toxins (SEG to SEI, SElJ, SEK to SET, SElU, SElV, SElX, and SElY) [1, 144, 145]. All superantigens share common protein domains that contribute to their superantigenicity by binding to MHC class II molecules and TCRs although the detailed interactions vary. Superantigenicity comes from the ability of superantigens binding to outside of peptide binding groove of MHC class II molecules and variable regions of TCR β chains (Vβ), unlike conventional antigens specifically recognized by antigen recognition sites of TCRs. Binding to TCR Vβ sequences provides diverse levels of Vβ specificity [146-148]. Affinity to MHC class II molecules also varies depending on their structures. Some superantigens that contain only one binding site to MHC class II α chain show low affinity to MHC class II molecules while other superantigens can bind to α- and β-chain of MHC class II molecule with higher affinity. Although the range of Vβ specificity does not appear to determine the strength of superantigenicity, superantigens with high affinity to MHC class II molecules can elicit stronger immune responses [1]. Recent studies demonstrated that superantigens also bind to receptors other than TCR and MHC II. Most staphylococcal superantigens have a short 12-14 long-peptide sequence called dodecapeptide binding region between the domains mediating binding to the TCR and MHC II [149, 150]. This dodecapeptide binding region mediates interaction with CD40 on B cells, CD28 on T cells, and unknown receptors on

epithelial cells and provide co-stimulatory signals for inducing proinflammatory cytokines associated with toxic shock syndrome [1, 151].

1.3.2 Superantigen-triggered immune responses

Binding of superantigens evokes activation of T cells by signaling mainly through TCR and CD28. Signaling cascades downstream of TCR and CD28 eventually induce activation of transcription factors, such as NFAT, NF-κB and AP-1, leading to upregulation of proinflammatory cytokines expression, such as IL-2 and IFN-γ [152]. Superantigens simultaneously activate APCs such as monocytes and macrophages to produce IL-1β and TNF-α which augment proinflammatory responses in concert with T cell responses [152]. Conventional antigens generally lead to activation of less than 0.01 % of T cell population in the periphery whilst superantigens can activate 20-30 % of T cells due to its ability to bind to TCR Vβ sequences, independent of the antigen recognition site [147]. Therefore, superantigens evoke massive cytokine production from activated T cells and APCs, in turn generating overwhelming immune responses. This is responsible for the clinical symptoms caused by superantigens, especially in the form of toxic shock syndrome accompanying symptoms such as hypotension, fever, and rash [2]. Eventually, activated T cells undergo activation-induced cell death and depletion of superantigen-reactive T cells, leading to unresponsiveness of the immune system against second introduction of superantigens. Also, the remaining superantigen-reactive T cells become unresponsiveness to a secondary superantigen challenge, which is termed as anergy [153]. This is a classically understood mechanism of superantigen-mediated immunosuppression [146, 154].

1.3.3 Superantigens and regulatory T cells

In addition to depletion of reactive T cells following the extensive immune reaction as described above, previous studies revealed that repeated injection of bacterial superantigens could induce specific T cell unresponsiveness achieved by IL-10 and/or TGF-β producing T cells. Injection of SEB resulted in unresponsiveness against secondary injection, conferred by suppressive CD8+ T cells [155]. These cells suppressed proliferation of SEB-reactive T cells, indicating the specificity of suppresion along with the finding of Jiang et al. which showed that CD8+ T cells after SEB injection specifically deleted CD4+ T cells bearing SEB-reactive TCR Vβ sequences [156]. Feunou et al. demonstrated that repeated injection of SEB induced CD4+CD25- T cells that can suppress SEB-specific primary T cells responses [157]. Immunosuppression conferred by CD4+CD25- T cells was independent with CD4+CD25+ tTregs since transfer of unresponsive CD4+CD25- T cells protected the thymectomized mice from SEB-induced shock. They also demonstrated that immunosuppresion by CD4+CD25+ Tregs induced after repeated injection of SEB was mediated by CTLA-4, enhancing expression and function of IDO in DC [86]. Noel et al. showed that unresponsive CD4+ T cells generated after repetitive injection of SEB suppresed the production of IL-2 and IFN-γ following SEB treatment *in vivo* and *in vitro*, mediated by IL-10 [7]. In another study using transgenic mice that have TCR repertories limited specifically to SEA or SEB, CD4+CD25+ T cells and CD4+CD25- T cells isolated from the mice after superantigens treatment were functionally suppressive and the suppession was strictly contact-dependent [8]. The authors also demonstrated that CD4+CD25- T cells could acquire regulatory properties after supernantigen stimulation in the absence of tTregs. In

the report of Miller et al., *in vivo* SEA-challenged CD4+ T cells produced IL-10 and TGF-β that inhibited IL-2 production in naïve responder T cells [158].

In vitro stimulation with superantigens also induce a subset of T cells with regulatory function. Taylor et al. demonstrated that stimulation of human PBMCs with 1ng/ml of staphylococcal and streptococal superantigens could induce funtional CD4+CD25+FOXP3+ and CD8+CD25+FOXP3+ Tregs [10, 119]. Stimulation of human PBMCs with SEB (3 μg/ml) could induce CD8+ Tregs with strong contact-dependent suppressive activity [9]. However, the effect of superantigen concentration was not demonstrated in these studies.

Collectively, Tregs appear to be invovled in superantigen-induced unresponsiveness of immune responses after superantigen exposure. Yet the mechanisms of superantigen-mediated Tregs induction regarding superantigen dose relavant to biological conditions and direct link between colonized *S. aureus* and superantigen-mediated immuosuppression has not been demonstrated.

1.4 Objective and significance of this dissertation

S. aureus commonly colonizes skins and mucosal membranes and sudden development of severe invasive staphylococcal infection can occur in immunocompromised patients or even in healthy individuals. However, the mechanism of pathogenesis is not well established yet. In this study, we aimed to identify the molecular mechanisms by which superantigens produced by colonized *S. aureus* contribute to pathogenesis of staphylococcal infection with following objectives and significance:

First, characterize concentration-dependent effect of superantigens. The role of superantigens has been focused on toxic shock syndrome or food poisoning which is hardly related to onset of invasive staphylococcal diseases. Colonized *S. aureus* produces relatively low concentration of superantigens, therefore, demonstrating how small quantity of superantigens contribute to the immune response could provide understanding of superantigen-mediated staphylococcal pathogenesis.

Second, identify development of CD8+ regulatory T cells by superantigens. Superantigens bind to and activate CD4+ T cells in TCR Vβ-specific manner and this is well described. In contrast, although CD8+ T cells respond to superantigens and proliferate as well, the knowledge of how superantigens interact with CD8+ T cells is lacking. Hence, studying CD8+ T cell biology in response to superantigens could advance our knowledge on staphylococcal superantigens.

Third, identify the suppression mechanisms by which superantigen-induced Tregs inhibit effector T cell proliferation. The mechanisms can vary depending on Treg induction condition and species (human/mouse). Therefore, identifying the suppression mechanisms could provide knowledge for therapeutic strategies in *S. aureus* infection.

Lastly, identify the concentration-dependent effects of superantigen in molecular level. Although the importance of antigen concentration on T cell differentiation has been described, the molecular mechanisms remain elusive. Studies on signaling pathways have been performed in mouse models, but the parallel study in humans is limited. Also, due to differences in humans and mouse, especially FOXP3 biology, identification of signaling pathways and FOXP3 is necessary in humans. Furthermore, this approach could advance our understanding on superantigen-induced immune responses on the molecular level.

CHAPTER II

INDUCTION OF IMMUNOSUPPRESSIVE CD8+CD25+FOXP3+ REGULATORY T CELLS BY SUBOPTIMAL STIMULATION WITH STAPHYLOCOCCAL ENTEROTOXIN C1 [159]

2.1 Abstract

Superantigens (SAgs) produced by *S. aureus* induce proliferation of T cells bearing specific TCR Vβ sequences and massive cytokinemia that cause toxic shock syndrome at high concentrations. However, the biological relevance of SAgs produced at very low concentrations during asymptomatic colonization or chronic infections is not understood. In this study, we demonstrate that suboptimal stimulation of human PBMCs with a low concentration (1 ng/ml) of staphylococcal enterotoxin C1 (SEC1), at which half-maximal T cell proliferation was observed, induced CD8+CD25+ T cells expressing markers related to regulatory T cells (Tregs) such as IFN-γ, IL-10, TGF-β, FOXP3+, CD28+, CTLA4+, TNFR2+, CD45RO+, and HLA-DR+. Importantly, these CD8+CD25+ T cells suppressed responder cell proliferation mediated by contact-dependent and soluble factor-dependent manners, involving galectin-1 and granzymes, respectively. By contrast, optimal stimulation of human PBMCs with a high concentration (1 μg/ml) of SEC1, at which maximal T cell proliferation was observed, also induced similar expression of markers related to Tregs including FOXP3 in

CD8+CD25+ cells, but these T cells were not functionally immunosuppressive. We further demonstrated that SAg induced TCR Vβ- and MHC II-restricted expansion of immunosuppressive CD8+CD25 T cells is independent of CD4+ T cells. Our results suggest that the concentration of SAg remarkably affects the functional characteristics of activated T cells, and low concentrations of SAg produced during asymptomatic colonization or chronic *S. aureus* infection induce immunosuppressive CD8+ regulatory T cells, potentially promoting colonization, propagation, and invasion of *S. aureus* in the host.

2.2 Introduction

Staphylococcus aureus causes some of the most critical infectious disease problems in the United States [160]. Annually, *S. aureus* accounts for approximately 5,000 cases of toxic shock syndrome (TSS), 70,000 cases of pneumonia, 40,000 cases of infective endocarditis (IE) and more than 500,000 post-surgical infections, resulting in 12,000 fatalities. Moreover, the increasing occurrence of methicillin resistant *S. aureus* (MRSA) with reduced sensitivity to vancomycin urgently demands alternative prevention and treatment strategies [161]. *S. aureus* frequently colonizes skin and mucosal membranes of the host without any clinical symptoms, but can suddenly erupt into a highly lethal invasive disease such as necrotizing pneumonia and IE in immunocompromised patients in hospital settings and even in healthy persons in the community [1, 162]. Efforts have been made to elucidate the mechanism of occurrence of highly lethal invasive disease by *S. aureus* in healthy community populations, but such mechanisms still remain elusive.

Staphylococcal enterotoxins (SEs), SE-like (SEl) toxins, and TSS toxin-1 (TSST-1) are superantigens (SAgs). Most SAgs bind outside the peptide binding grooves of MHC II molecules on APCs and specific variable regions of TCR β chains (Vβ) on T cells (SAg-reactive T cells) [163, 164]. Binding in this manner activates APCs and induces extensive TCR Vβ-dependent proliferation of T cells, causing high level secretion of proinflammatory cytokines such as IL-1, IL-2, IFN-γ, and TNF-α and immunomodulatory cytokines such as IL-10 and TGF-β [165]. The initial expansion of T cells is followed by activation-induced cell death or apoptosis, leading to clonal deletion of SAg-reactive T cells [163, 166]. SAg-reactive T cells that escape from clonal deletion fail to proliferate and secrete IL-2. This phenomenon is often referred to as anergy [167]. Thus far, 25 SAgs including SEA through SElX (except F) and TSST-1 have been characterized in *S. aureus* and most clinical isolates typically carry 5-7 different SAg genes [1, 144]. The causal link of SAgs in the pathogenesis of toxic shock syndrome and food poisoning, in which large amounts of SAgs (1-100 μg/ml) are produced, is well established [1, 168]. However, relatively small concentrations of SAgs (0.65 – 5 ng/ml) are produced in more frequently occurring asymptomatic colonization or chronic *S. aureus* infections [169-171], but the biological relevance of such small concentrations of SAgs in the pathogenesis of *S. aureus* is not fully understood.

During infection, it is crucial to activate innate and adaptive immunity to control the pathogen, but it is equally important to regulate innate and adaptive immune responses to prevent tissue damage. Tregs have been recognized as a key component in the maintenance of immunological self-tolerance and the control of T cell immunity to prevent tissue damage by an extended proinflammatory response [172]. However,

immunosuppression by Tregs could be exploited by pathogens to promote infections [173, 174]. The Tregs can be broadly classified into CD4+ and CD8+ Tregs. CD4+ Tregs have been characterized as thymus-derived CD4+CD25+FOXP3+T cells (tTreg), and they can be induced by peripheral conversion of CD4+CD25- conventional T cells into CD4+CD25+FOXP3+T cells (pTreg) or *in vitro*-induced CD4+CD25+FOXP3+T cells (iTreg) by stimulation of peripheral blood mononuclear cells (PBMCs) via TCR using anti-CD3 mAb and anti-CD3/CD28 beads [172, 175-177].

CD8+ Tregs were first described as CD8+ suppressor T cells from a mouse study in 1970 [23] showing the adaptive transfer of tolerance. Recently, CD8+ Treg studies have been rekindled because of the crucial roles of CD8+ Tregs in autoimmune disease and immunosuppression in transplant recipients. Several studies revealed various subsets of CD8+ Tregs. For example, in humans, repetitive stimulation of $CD8^+$ T cells with allogeneic or xenogeneic APC induce $CD8^+CD28^-$ T cells expressing immunoglobulin-like transcript (ILT)-3 and ILT-4, which render tolerance in APC [117, 178]. Alloantigenic stimulation of $CD8^+$ T cells with CD3 and CD28 mAbs induces $CD8^+CD103^+$ T cells, which suppress effector T cells by a cell-to-cell contact-dependent mechanism [179]. A recent study demonstrated that stimulation of human PBMCs with staphylococcal SAg (SEA and TSST-1) and streptococcal SAgs (SPEA, K/L) at a low concentration (1 ng/ml) induced CD8+CD25+FOXP3+ T cells with suppressive activity mediated by an unknown cell-to-cell dependent mechanism [119]. In line with these findings, our previous study demonstrated that stimulation of bovine PBMCs with SEC1 at a concentration resulting in suboptimal T cell proliferation (1-5 ng/ml) induced immunosuppressive CD4+CD25+ and CD8+CD25+ Tregs [170]. These results suggest

that the strength of TCR signaling determined by the concentration of SAg might differentially regulate phenotypic and functional characteristics of immunosuppressive T cells.

In this study, we determined the phenotypic and functional characteristics of human CD8+CD25+ T cells induced by stimulation of human PBMCs with SEC1 at concentrations inducing optimal (1 μg/ml) and suboptimal (1 ng/ml) stimulation. Our results demonstrated that despite similar expression of phenotypic and functional Treg markers in CD8+CD25+FOXP3+ T cells induced from both optimal and suboptimal stimulation, suppressive activity was only observed in CD8+CD25+FOXP3+ T cells induced from suboptimal stimulation. These results suggest that a low concentration of SAg produced during asymptomatic colonization or chronic *S. aureus* infection induces immunosuppressive CD8+ Tregs that may trigger outbreaks of highly lethal invasive *S. aureus* infections in healthy community populations as well as immunocompromised hospital patients.

2.3 Materials and methods

2.3.1 PBMC isolation

Healthy volunteers aged 18-40 y of age were recruited into the study. The protocol (13-191) was reviewed and approved by the institutional review board at Mississippi State University, and a written informed consent form was obtained from all volunteers. Blood was obtained by venipuncture, and peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Histopauqe-1077 (Sigma-Aldrich, St. Louis, MO). The isolated PBMCs were resuspended in RPMI 1640 medium

containing 10% heat-inactivated FBS, 2 mM L-glutamine and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

2.3.2 Purification of SEC1

Dr. Scott Minnich (University of Idaho) kindly provided staphylococcal enterotoxin C1 (SEC1) which was purified from *S. aureus* RN4220 (pMIN121) using preparative isoelectric focusing with a broad isoelectric point (3 to 10) and a narrow isoelectric point (6 to 8) range of ampholytes in succession [180].

2.3.3 Cell stimulation

To determine T cell proliferation in response to SEC1, human PBMCs were adjusted to 5×10^5 cells per well in a 96-well plate with the indicated concentrations of SEC1 (0.001-10,000 ng/ml). Cells were incubated at 37°C in 5% CO_2 for 72 hours. Cells were pulsed with 1 μCi of [^{3}H]-thymidine for 18 hours. Cellular DNA was harvested on glass fiber filters, and incorporation of [^{3}H]-thymidine into cellular DNA was quantified by liquid scintillation counting. For phenotypic and functional assay, CD25+ cells were depleted from the PBMCs using CD25 microbeads (Miltenyi Biotec, San Diego, CA) to prevent expansion of preexisting Tregs (CD4+CD25+ and CD8+CD25+ T cells) and their influence on induction of Tregs by SEC1. The PBMCs depleted of CD25+ cells (PBMC/CD25-) were adjusted to 1.5×10^6 cells per well in 6-well plates and stimulated with SEC1 at optimal (1 μg/ml) or suboptimal (1 ng/ml) stimulation conditions for the indicated times.

2.3.4 Immunophenotyping and flow cytometry

For phenotypic characterization of CD8+CD25+ T cells induced by SEC1 stimulation, mAbs specific to human CD8-FITC, CD25-PECy7, CTLA-4-allophycocyanin, IL-10- allophycocyanin, and IFN-γ- allophycocyanin were purchased from BD Biosciences (San Jose, CA). Antibodies specific for human TCR Vβ2-PE and TCR Vβ14-PE were purchased from Beckman Coulter (Brea, CA). MAbs specific for human CD28-PE, CD127- allophycocyanin, FOXP3- allophycocyanin, GITR-PE, HLA-DR-PE, OX40-PE, and TNFR2-PE as well as Brefeldin A were purchased from eBioscience (San Diego, CA). Mab specific for anti-TGF-β- allophycocyanin was purchased from R&D Systems (Minneapolis, MN). For neutralization assays, anti-human IL-10, TGF-β, IFN-γ, and isotype controls for rat-IgG1 and mouse-IgG1 were purchased from eBioscience. To measure cytokine secretion, culture supernatants from suboptimal stimulation with SEC1 were harvested every 2 days and analyzed by a Milliplex 34-plex human cytokine Luminex kit (Millipore) according to the manufacturer's instruction.

For surface staining, cells were harvested, washed with FACS buffer (eBioscience), and stained for 30 min on ice with different combinations of mAbs specific for human CD8-FITC, CD25-PECy7, CD28-PE, CD127- allophycocyanin, GITR-PE, HLA-DR-PE, OX40-PE, TNFR2-PE, TCR Vβ2-PE, or TCR Vβ14-PE. For intracellular staining, cells were incubated with Brefeldin A for 1 hour under previously described culture conditions, followed by surface staining, and then further fixed and permeabilized with the Human FOXP3 Buffer Set (eBioscience) according to the manufacturer's instructions. Fixed/permeabilized cells were stained with mAbs specific for human FOXP3- allophycocyanin, TGF-β- allophycocyanin, CTLA-4-

allophycocyanin, IL-10- allophycocyanin, or IFN-γ- allophycocyanin. Cells were washed with FACS buffer and analyzed using a FACSAria III flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one (FMO) controls or isotype controls were used to ensure that the upregulation was not due to a non-specific Ab effect.

2.3.5 Autologous mixed leukocyte reaction (MLR)

For functional assays, CD8+CD25+ or CD8+CD25- T cells were purified by negative selection with a CD8+ T cell isolation kit II, followed by anti-CD25 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. For some assays, naïve CD4+CD25- T cells and CD14+ cells were purified from unstimulated PBMCs using a naïve CD4+ T cell purification kit and a monocyte purification kit, respectively (Miltenyi Biotec). The purity of isolated cells was routinely monitored by flow cytometry, and was consistently over 95-99% (data not shown).

Naïve CD4+CD25- T cells were labeled with 2.5 μM CFSE (Invitrogen, Carlsbad, CA) for 5 min at 37 ^{o}C and were washed with warm RPMI 1640 medium. CFSE-labeled naïve CD4+CD25- T cells (1×10^5 cells per well, responder cells) were mixed with various numbers (1×10^5, 5×10^4, 1×10^4) of CD8+CD25+ T cells induced from suboptimal and optimal stimulation with SEC1 for 6 and 4 days, respectively. Cells were stimulated with anti-CD3/CD28 beads at a 1:1 bead to cell ratio. After 4 days, cells were labeled with CD4-PE to separate the CFSE-labeled CD4+ T cells from CD8+CD25+ T cells induced by SEC1 stimulation. Proliferation of CD4+ T cells was determined as the dilution of CFSE signal using flow cytometry. For the contact dependent suppression assay, CD8+CD25 T cells induced from suboptimal stimulation

with SEC1were treated with 10 μM of mitomycin C (Sigma-Aldrich) for 2 hours to prevent protein synthesis and proliferation [181, 182]. After thorough washing, mitomycin C-treated CD8+CD25+ T cells were mixed with CFSE labeled responder cells and stimulated with anti-CD3/CD28 beads as described above. For the soluble factor dependent suppression assay, CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 were added to the upper chamber of a transwell (0.45 μM pore size, Corning Life Sciences, Tewksbury, MA) and an equal number of CFSE labeled responder cells were added to the bottom chamber of a transwell. Cells were stimulated as described above.

In some experiments, mAbs (10 μg/ml) specific for CTLA-4 (eBioscience), IL-10 (R&D systems), TGF-β (R&D systems), IFN-γ (eBioscience), or Gal1 (Santa Cruz, Dallas, TX), or the same quantify of isotype controls (eBioscience) were added to cells. In addition, ZM241385 (10 μM, adenosine 2A antagonist, Tocris, Minneapolis, MN), indomethacin (25 μM, COX inhibitor, Sigma-Aldrich), or 3,4-dichloroisocumarin (50 μM, DCI, serine protease inhibitor, EMD Millipore, Billerica, MA) were added to the autologous MLR assay as indicated in the results.

2.3.6 Immunoblotting

To determine activation of CD8+ T cells by SEC1 via signaling through TCR, we determined the phosphorylation of CD3ζ. CD8+CD25- T cells (5×10^5) that were purified and stimulated with 1 ng/ml of SEC1 for 10 minutes in the presence or absence of CD14+ monocytes (4×10^5). Whole cell lysates were separated by 12.5% SDS-PAGE and transferred to a PVDF membrane. The phosphorylation of CD3ζ was detected by anti-CD3ζ (phosphoryl Y142) Ab (Abcam, Cambridge, MA), followed by HRP-

conjugated sheep anti-mouse IgG (GE Healthcare). The loading control was assessed by immunoblot against β-actin using anti-β actin Ab (Sigma-Aldrich), followed by HRP-conjugated donkey anti-rabbit IgG (GE Healthcare).

2.3.7 Quantification of selective expansion of TCR Vβ using real-time PCR.

Total RNA was prepared from samples taken before and after stimulation of CD8+CD25- T cells with SEC1 (1 ng/ml) for 4 days and cDNA was synthesized using a cDNA synthesis kit (Invitrogen). Selective expansion of TCR Vβ was assessed by quantitative real-time PCR using an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA) as described previously (29).

2.3.8 Statistical analysis

Data were obtained from 3 separate experiments for each of 2 or 3 donors generating 6 or 9 independent experiments. Statistical significance among different treatment groups was analyzed with the one-way analysis of variance (ANOVA), followed by Tukey's HSD for post hoc test using GraphPad Prism software (Version 7.0.3. La Jolla, CA). Differences with p value < 0.001 were considered statistically significant.

2.4 Results

2.4.1 Stimulation of human PBMCs by SEC1 induced expression of FOXP3 in CD8+ T cells

We first measured human T cell proliferation in response to a serial dilution of SEC1 to determine the concentrations of SEC1 needed to induce maximal and half maximal T cell proliferation to set as the optimal and suboptimal stimulation conditions,

respectively. As expected, the T cell proliferation response was positively related to the concentration of SEC1 (Fig. 2.1A). Maximal T cell proliferation was observed at 1 µg/ml and approximately half maximal T cell proliferation was observed at 1 ng/ml. The decreased proliferation observed at 10 µg/ml was likely due to the depletion of culture medium components resulting from vigorous proliferation. Based on these results, we defined the concentration of SEC1 that induced optimal and suboptimal stimulation as 1 µg/ml and 1 ng/ml, respectively.

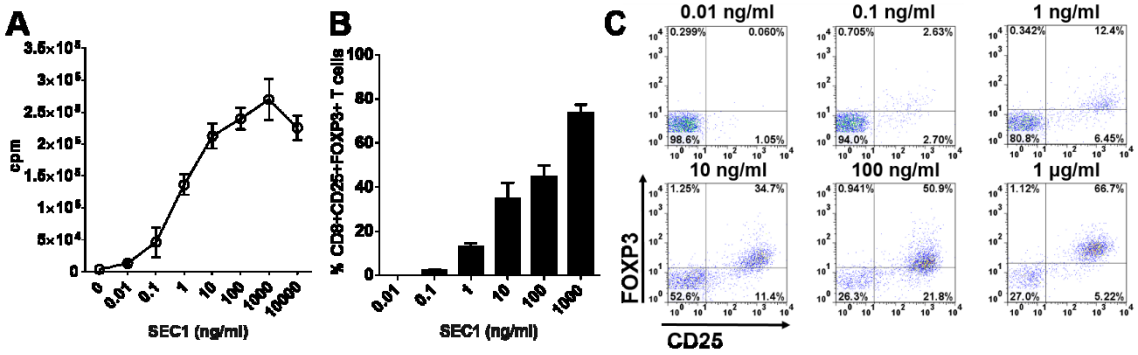

Figure 2.1 Concentration-dependent expression kinetics of FOXP3 in CD8+CD25+ T cells by SEC1.

(A) Human T cell proliferation after exposure to SEC1 for 4 days was measured by the incorporation of [^{3}H]-thymidine. The data shown are the mean ± SEM of nine independent experiments (three separate experiments for each of three different donors). (B) Human PBMCs were stimulated with various concentrations of SEC1 for 4 days, and expression of CD25 and FOXP3 in CD8+ T cells was measured by flow cytometry. The data shown are from a single representative experiment. (C) The data shown are the mean percentage ± SEM of CD8+CD25+FOXP3+ T cell combined from nine independent experiments.

To examine the kinetics of SEC1 concentration-dependent changes in expression of FOXP3 in human CD8+CD25+ T cells, human PBMC depleted of CD25+ cells (PBMC/CD25-) were stimulated with various concentrations of SEC1 ranging from 0.01 ng/ml to 1 µg/ml for 4 days. The CD25+ T cells were depleted to prevent expansion of preexisting regulatory cells commonly expressing CD25. Expression of FOXP3 in

CD8+CD25+ T cells was positively related to the concentration of SEC1. Expression of FOXP3 in CD8+CD25+ T cells was the highest at 1 μg/ml and gradually decreased with lower concentrations of SEC1 (Fig. 2.1B and C). To examine the kinetics of expression of FOXP3 in CD8+CD25+ T cells, human PBMC/CD25- cells were stimulated with SEC1 under optimal (1 μg/ml) and suboptimal (1 ng/ml) stimulation conditions up to 8 days. Under the optimal stimulation condition, expression of FOXP3 in CD8+CD25+ T cells rapidly increased and peaked at day 4 post stimulation (62.4%±11.5), then gradually decreased thereafter (Fig. 2.2A and B, optimal panel). On the contrary, under the suboptimal stimulation condition, expression of FOXP3 in CD8+CD25+ T cells was delayed but gradually increased to reach 54.35%±6.5 at day 8 post stimulation (Fig. 2.2A and B, suboptimal panel).

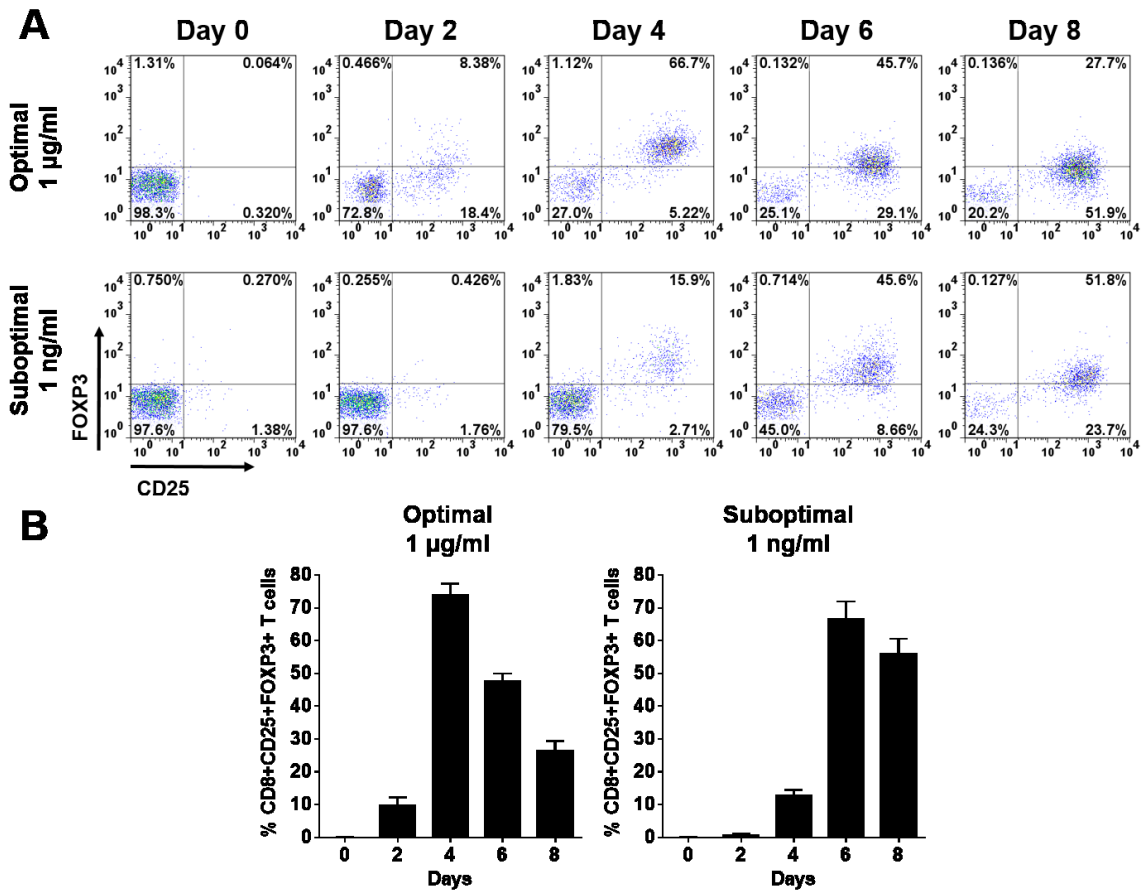

Figure 2.2 Time-dependent expression kinetics of FOXP3 in CD8+CD25+ T cells by induced by stimulation with SEC1.

Human PBMCs were stimulated with an optimal (1 µg/ml) and a suboptimal stimulation condition (1 ng/ml) of SEC1 up to 8 days. Expression of CD25 and FOXP3 in CD8 T cells was measured using flow cytometry. (A) Data shown are a single representative experiment gated on CD8+ T cells. (B) Data shown are the mean percentage ± SEM of CD8+CD25+FOXP3+ T cell combined from nine independent experiments measured from three different donors.

2.4.2 CD8+CD25+ T cells induced from the suboptimal, but not optimal, SEC1 stimulation condition were functionally immunosuppressive

To determine functional characteristics of CD8+CD25+ T cells induced from optimal and suboptimal stimulation with SEC1, we conducted autologous mixed leukocyte reaction (MLR). The PBMC depleted of CD25+ cells were stimulated with 1 µg/ml (optimal stimulation) or 1 ng/ml (suboptimal stimulation) of SEC1 for 4 days or 6

days at which FOXP3 was highly expressed in CD8+CD25+ T cells. Purified CD8+CD25+ T cells were co-cultured with freshly prepared autologous CFSE-labeled naïve CD4+CD25- T cells (responder cells) in the presence of anti-CD3/CD28 beads as T cell stimulants. To separate the responder cells from CD8+CD25+ T cells, cells were stained with CD4-PE and CD4+ T cells were gated as depicted in Appendix Fig. 1. As expected, anti-CD3/CD28 beads induced proliferation of naïve CD4+CD25- T cells (Fig. 2.3A). Proliferation of naïve CD4+CD25- T cells was significantly inhibited by CD8+CD25+ T cells induced by suboptimal stimulation with SEC1. The suppression was positively related to the number of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 (Fig. 2.3B and E). By contrast, CD8+CD25+ T cells induced from optimal stimulation with SEC1 slightly increased proliferation of naïve CD4+CD25- T cells (Fig. 2.3C and E). In the absence of anti-CD3/CD28 beads, CD8+CD25+ T cells induced by optimal stimulation with SEC1 did not induce proliferation of naïve CD4+CD25- T cells indicating there was no SEC1 contamination in CD8+CD25+ T cells purified after stimulation with SEC1 (Fig. 2.3C). Together these results demonstrate that CD8+CD25+ T cells induced by suboptimal stimulation with SEC1, but not optimal stimulation, are functionally immunosuppressive.

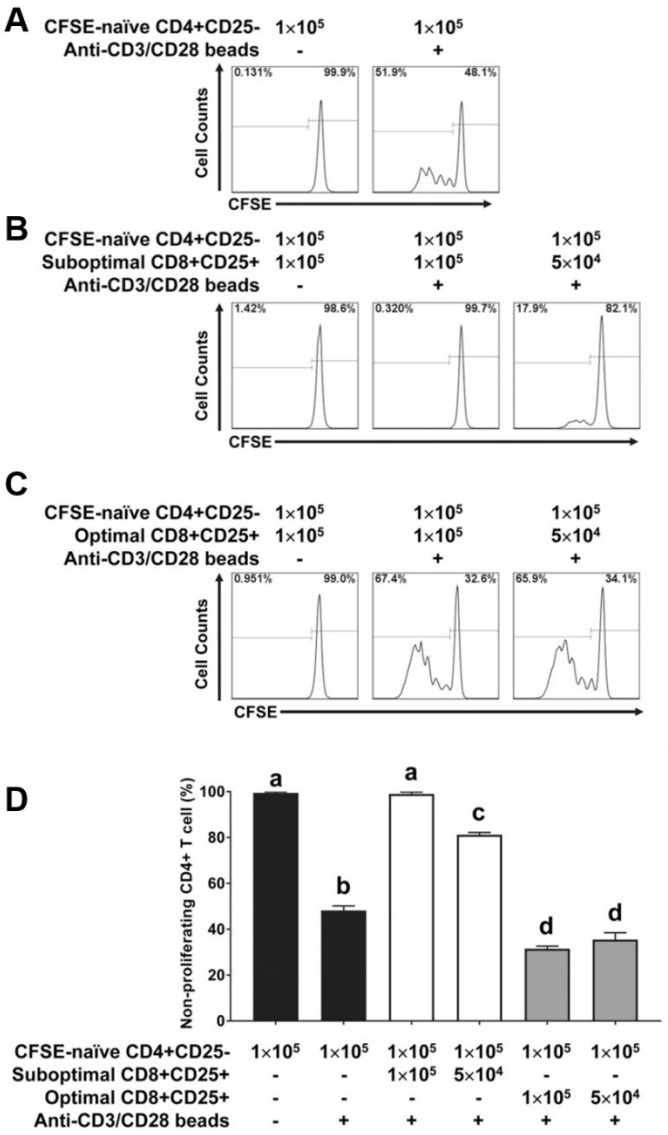

Figure 2.3 Suppression of naïve CD4+CD25- T cell proliferation by CD8+CD25+ T cells induced from a suboptimal stimulation with SEC1, not from an optimal stimulation.

(A) Naïve CD4+CD25- T cells were purified, labeled with CFSE, and stimulated anti-CD3/CD28 beads for 4 days. Proliferation of CD4+ T cells were analyzed by quantifying the dilution of CFSE signal measured by flow cytometry. (B) CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 for 6 days were purified and co-cultured with CFSE-labeled CD4+CD25- T cells at various ratios for 4 days in the absence or presence of anti-CD3/CD28 beads. (C). CD8+CD25+ T cells induced from optimal stimulation with SEC1 for 4 days were purified and co-cultured with CFSE-labeled CD4+CD25- T cells for 4 days in the absence or presence of anti-CD3/CD28 beads. (E) The data shown are the mean ± SEM of non-proliferating CD4+ T cells combined from nine independent experiments measured from three different donors. Different letters indicate significant differences in the mean percentage between treatments determined by ANOVA followed by Tukey's HSD test ($p < 0.001$).

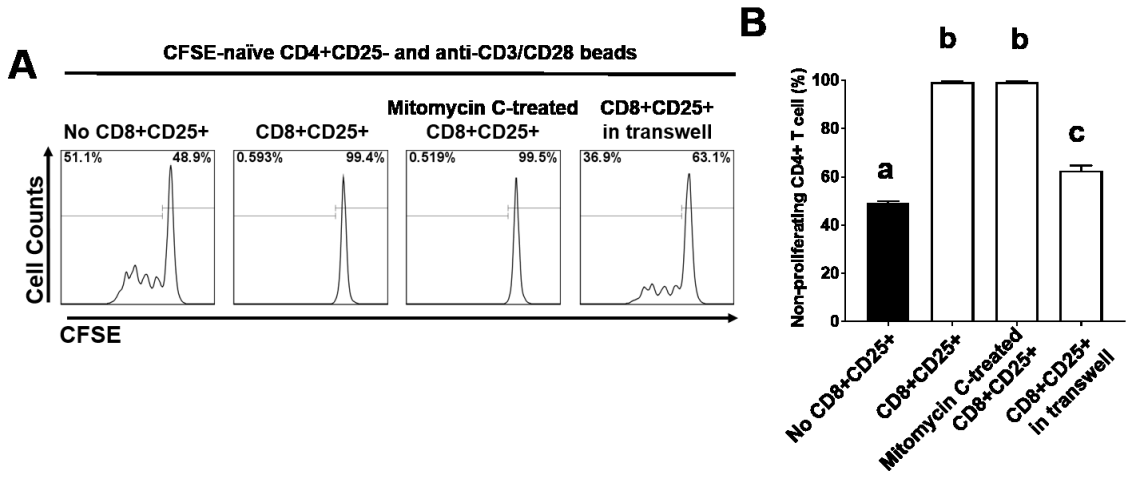

Figure 2.4 Suppression by CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 is mediated by the cell-to-cell contact dependent manner to a larger extent than the soluble factor-dependent manner.

CFSE-labeled naive CD4+ T cells were co-cultured with an equal number of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 fixed with mitomycin C for a contact-dependent suppression assay or with CD8+CD25+ T cells in the top chamber of Transwell for a soluble factor-dependent suppression assay. Cells were stimulated with anit-CD3/CD28 beads and proliferation of CD4+ T cells were analyzed by flow cytometry. (A) Data shown are a single representative result of CFSE dilution histograms. (B) Data shown are the mean percentage ± SEM of non-proliferating CD4+ T cells combined from nine independent experiments measured from three different donors. Different letters indicate significant differences in the mean percentage between treatments determined by ANOVA followed by Tukey's HSD test ($p < 0.001$).

To investigate whether the suppressive activity of CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 is mediated by cell-to-cell contact or soluble factors, we conducted autologous MLR using mitomycin C-treated CD8+CD25+ T cells to prevent de novo T cell activation or in a transwell to prevent direct cell-to-cell contact, but allow migration of soluble factors (for soluble factor-dependent suppression). The mitomycin C-treated CD8+CD25+ T cells completely inhibited proliferation of naïve CD4+CD25- T cells in response to anti-CD3/CD28 beads (Fig. 2.4). On the contrary, CD8+CD25+ T cells in a transwell partially inhibited proliferation of naïve CD4+CD25- T cells in response to anti-CD3/CD28 beads (Fig.2.4). These results suggest that the

suppressive activity by CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 is mediated predominantly by cell-to-cell contact, but also involves soluble factors.

2.4.3 CD8+CD25+FOXP3+ T cells induced by suboptimal stimulation with SEC1 expressed surface makers and cytokines related to regulatory cells

To characterize the phenotype of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 for 6 days, cellular markers related to Tregs were evaluated using flow cytometry. Before stimulation, naïve CD8+ T cells did not express classical Treg markers such as CD25, CTLA-4, GITR, OX-40, TNFR2, and CD45RO (Appendix Fig. 2A). After suboptimal stimulation, CD8+CD25+ cells highly expressed CD25, CD28, HLA-DR, CTLA-4, TNFR2, and CD45RO but expression of CD127, GITR, and OX40 decreased or slightly increased (Appendix Fig. 2A). To further analyze the phenotype of CD8+CD25+FOXP3+ T cells, analysis was performed by gating on CD8+CD25+ T cells. The CD8+CD25+FOXP3+ T cells highly expressed CD28, CD45RO, CTLA-4, HLA-DR, and TNFR2, and less than 20% of CD8+CD25+FOXP3+ T cells expressed CD127, GITR, and OX40 (Fig. 2.5A and Appendix Fig. 2B). It is noteworthy that CTLA-4 was only detected by intracellular staining, but not by surface staining. The CD8+CD25+ T cells induced from optimal stimulation with SEC1 showed similar phenotypes with the exception of higher expression of GITR and OX40 compared to suboptimal stimulation (Appendix Fig. 3)

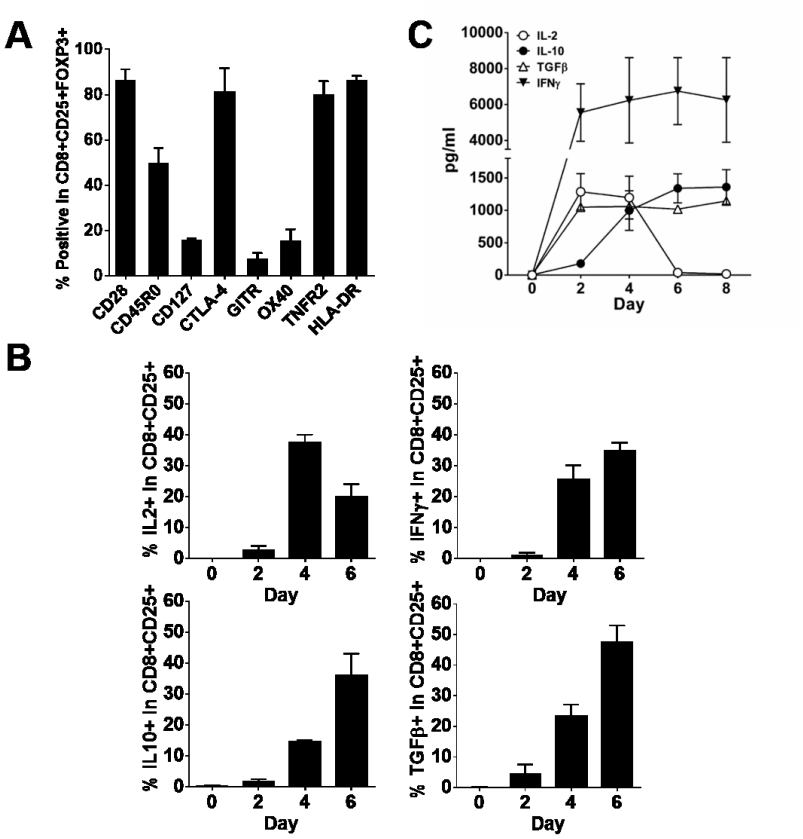

Figure 2.5 Expression of cell surface markers and cytokines related to Tregs by CD8+CD25+ T cells induced from suboptimal stimulation with SEC1.

Human PBMC depleted of CD25+ T cells were stimulated with a suboptimal stimulation concentration of SEC1 (1 ng/ml) for 6 days. Expression of surface markers and cytokines related to related to Tregs were measured by flow cytometry and a Luminex ELISA kit. (A) Data shown are the mean percentage ± SEM of CD8+CD25+FOXP3+ T cells expressing surface markers related to Tregs combined from nine independent experiments measured from three different donors. Expression of CTLA-4 was measured by the intracellular staining. (B) Data shown are the mean percentage ± SEM of CD8+CD25+ T cells expressing cytokines related to Tregs measured by intracellular staining and flow cytometry. Data are combined from nine independent experiments measured from three different donors. (C) During suboptimal stimulation with SEC1, the culture supernatant was collected every 2 days and analyzed by a Milliplex Luminex cytokine analysis kit. Data shown are combined results from six independent experiments measured from two different donors.

To determine the functional characteristics of CD8+CD25+ T cells induced by suboptimal stimulation with SEC1, expression of cytokines related to regulatory functions such as IL-2, IL-10, TGF-β, and IFN-γ were analyzed using flow cytometry and a Milliplex Luminex cytokine analysis kit. Before stimulation, minimal expression of IL-

2, IL-10, TGF-β, and IFN-γ in CD8+ T cells was detected (Fig. 2.5B). Upon suboptimal stimulation, IL-2 gradually increased and peaked at day 4, then gradually decreased at day 6. Expression of IL-10, TGF-β, and IFN-γ gradually increased by day 6 and was sustained. Notably, a decrease of IL-2 expression at 6 days (Fig. 2.5B) was concurrent with an increase of FOXP3 expression (Fig. 2.2), which is a known transcriptional repressor of the IL-2 promoter. Similar patterns of cytokine secretion in the culture media was observed using a Milliplex Luminex cytokine analysis kit (Fig. 2.5C). Taken together, the characteristics of CD8+CD25+FOXP3+ T cells induced from suboptimal stimulation with SEC1 was CD28+, CTLA4+, TNFR2+, CD45RO+, HLA-DR+, secreting IL-10, TGF-β, and IFN-γ.

2.4.4 Immunosuppression of CD8+ Treg induced from suboptimal stimulation with SEC1 was mediated by galectin-1 and granzymes

Previous studies demonstrated that immunosuppression by CD4+ and CD8+ Tregs is mediated by CTLA-4, IL-10, TGF-β, and IFN-γ via membrane bound receptors or direct interaction with APC and CD4+ T cells [172, 178, 179, 183, 184]. Since expression of these molecules was highly increased in CD8+CD25+ T cells induced by suboptimal stimulation with SEC1, we sought to determine their role in immunosuppression using neutralizing mAbs. The blockade of CTLA-4, IL-10, TGF-β, and IFN-γ using mAb failed to inhibit the suppressive activity of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 (Fig. 2.6). Various combinations of mAb against these proteins and cytokines also did not affect the suppressive activity (data not shown).

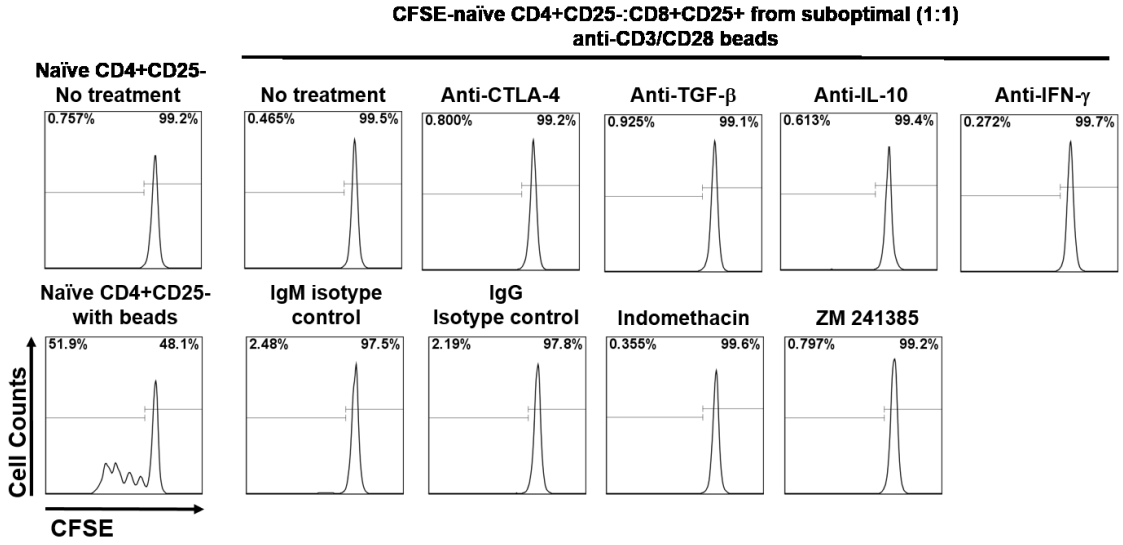

Figure 2.6 Suppression by CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 was not dependent on CTLA-4, TGF-β, IL-10, IFN-γ, CTLA-4, cAMP, and COX.

CFSE-labeled naïve CD4+CD25- T cells (1×10^5) were co-cultured with an equal number of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 in the presence of anti-CD3/CD28 beads and mAb (10 μg/ml) neutralizing CTLA-4, TGF-β, IL-10, IFN-γ, Indomethacin (25 μM, COX inhibitor), ZM241385 (10 μM, adenosine 2A antagonist) for 4 days. Proliferation of CD4+ T cells were analyzed by flow cytometry. Data shown are a single representative CFSE dilution histograms from nine independent experiments measured from three different donors.

Adenosine released by Tregs triggers accumulation of intracellular cAMP which induces the transcriptional factor ICER (inducible cAMP early repressor), resulting in suppression of T cells [185]. The blockade of adenosine receptor 2A by the small molecule inhibitor, ZM241385 [186], with an increasing dose up to 10 μM did not affect the suppressive activity of CD8+CD25+ T cells induced from suboptimal stimulation (data with 10 μM are shown in Fig. 6). A previous study showed that CD4+CD25+ Tregs express cyclooxygenase-2 and produce prostaglandin E2 which suppresses T cell immune responses by eliciting cAMP mediated immune suppression (34). However, the inhibition of COX by indomethacin with an increasing dose up to 25 μM did not affect the

suppressive activity of CD8+CD25+ T cells either (data with 25 µM are shown in Fig. 2.6).

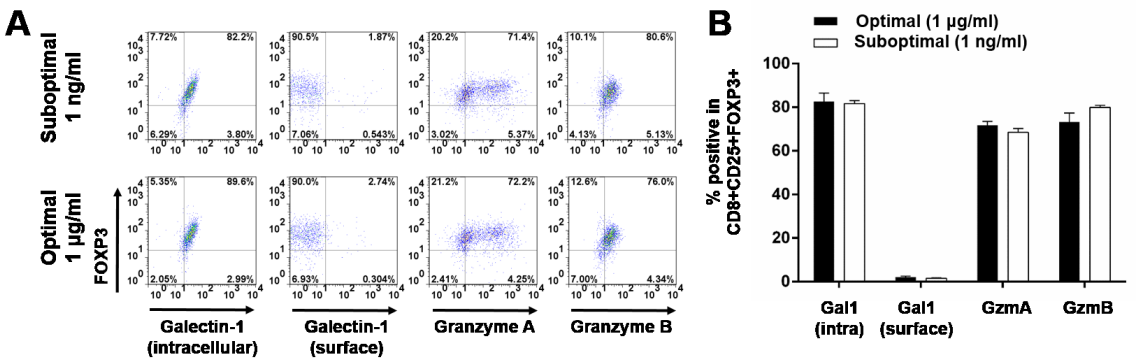

Figure 2.7 Expression of galectin-1 and granzymes by CD8+CD25+ T cells induced from optimal and suboptimal stimulation with SEC1.

(A) Human PBMC depleted of CD25+ T cells were stimulated with an optimal (1 µg/ml) and suboptimal stimulation concentration of SEC1 (1 ng/ml) for 4 and 6 days, respectively. Intracellular and surface expression of galectin-1 and intracellular expression of granzyme A and B was analyzed by flow cytometry. Data shown are a single representative dot plots gated on CD8+CD25+ T cells. (B) Data shown are the mean percentage ± SEM of CD8+CD25+FOXP3+ T cells expressing galectin-1, granzyme A and B combined from nine independent experiments measured from three different donors.

In an effort to elucidate phenotypic and functional markers of CD8+ Tregs, we performed transcriptomic and proteomic analyses and found that galectin-1 and granzyme A and B were consistently upregulated in CD8+CD25+ T cells induced by suboptimal stimulation with SEC1. To confirm the transcriptomic and proteomic analysis results, we measured expression of galectin-1 and granzymes in CD8+CD25+ T cells using flow cytometry. In both optimal and suboptimal stimulation conditions, galectin-1 was predominantly detected by intracellular staining, not by surface staining (Fig. 2.7A and B). Similarly, granzyme A and B were highly expressed intracellularly in CD8+CD25+ T cells (Fig. 2.7A and B). Finally, the blockade of galectin-1 with an anti-Gal-1 mAb or granzymes with a serine protease inhibitor (DCI, 3,4-dichloroisocumarin) partially

attenuated the suppressive activity of CD8+CD25+ T cells induced by suboptimal stimulation, restoring approximately 50% of proliferation as compared to the naïve CD4+ T cell control (Fig. 2.8). Combined treatment with DCI and anti-Gal1 restored approximately 75% of native T cell proliferation (Fig. 2.8). Furthermore, addition of anti-Gal-1 mAb to autologous MLR using mitomycin C-treated CD8+CD25+ T cells fully inhibited suppressive activity. In contrast, an addition of anti-Gal-1 mAb to autologous MLR in transwell did not inhibit the suppressive activity. These results strongly suggest that galectin-1 is largely responsible for the contact-dependent-suppression by CD8+CD25+ T cells induced from suboptimal stimulation with SEC1. Granzymes are well known soluble factors produced by cytotoxic CD8+ T cell and induce apoptosis in target cells [187]. Addition of DCI, a serine protease inhibitor, to the autologous MLR in the transwell fully inhibited the suppressive activity of CD8+ Treg. These results suggest that granzymes are responsible for the soluble factor-dependent suppression by CD8+CD25+ T cells induced from suboptimal stimulation with SEC1.

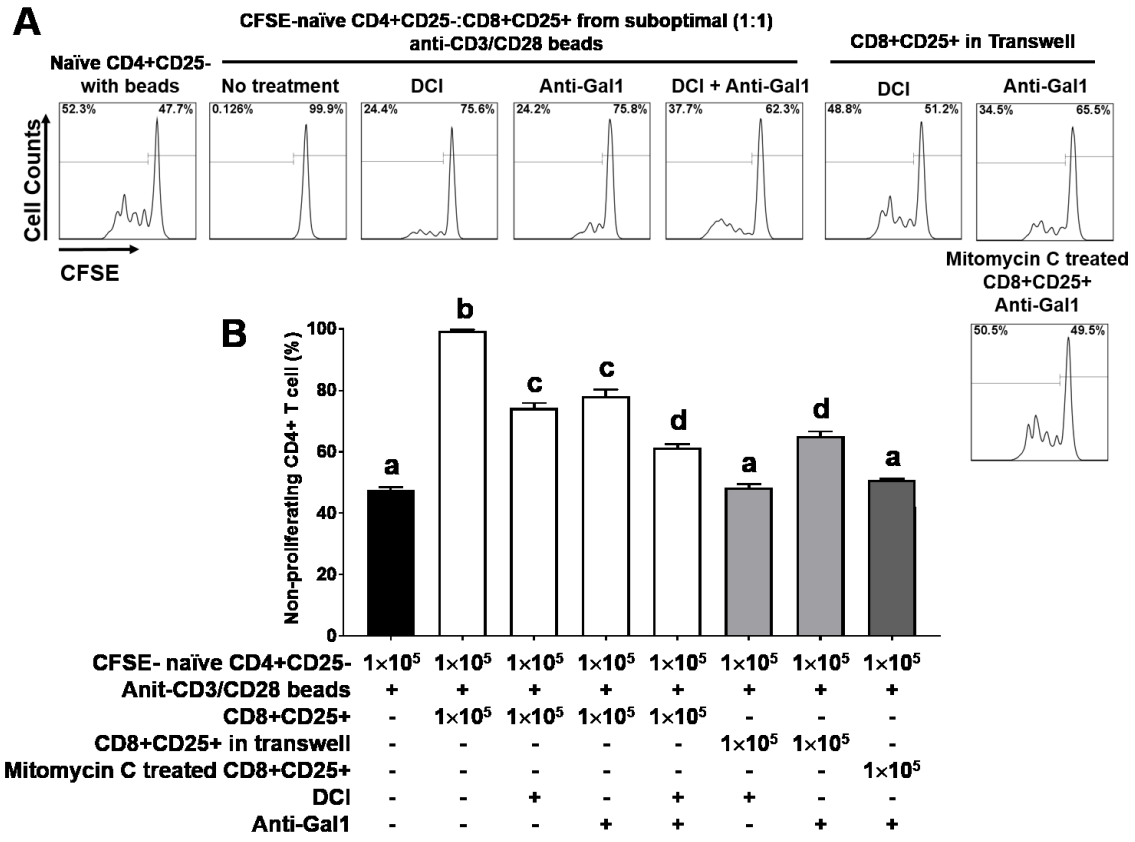

Figure 2.8 The suppressive activity of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 is mediated by galectin-1 and granzymes.

CFSE-labeled naïve CD4+CD25- T cells (1×10^5) were co-cultured with an equal number of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 in the presence of anti-CD3/CD28 beads and a mAb (10 µg/ml) neutralizing galectin-1 and/or 3,4-dichloroisocumarin (50 µM, DCI, serine protease inhibitor) for 4 days. In some experiments, CD8+CD25+ T cells induced from suboptimal stimulation with SEC1 were added to the top chamber of the Transwell or were fixed with mitomycin C and added to the co-culture. Proliferation of CD4+ T cells were analyzed by flow cytometry. (A) Data shown are a single representative CFSE dilution histograms. (B) Data shown are the mean percentage ± SEM of non-proliferating CD4+ T cells combined from nine independent experiments measured from three different donors. Different letters indicate significant differences in the mean percentage between treatments determined by ANOVA followed by Tukey's HSD test ($p< 0.001$).

2.4.5 Induction of CD8+CD25+FOXP3 T cells by a suboptimal stimulation with SEC1 is APC-restricted and TCR-Vβ dependent manner

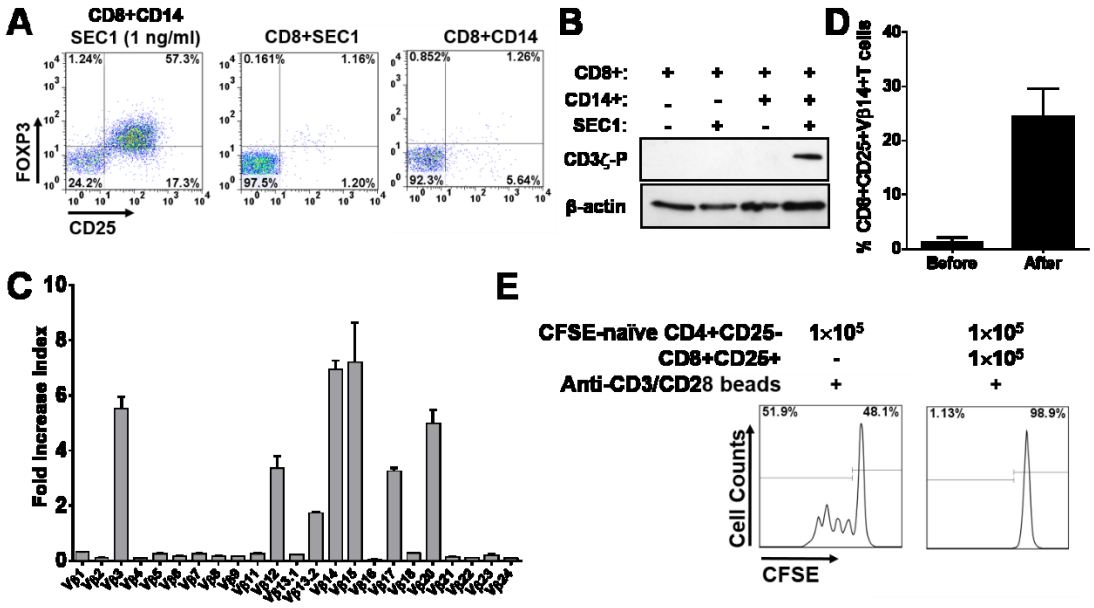

Figure 2.9 Induction of CD8+CD25+FOXP3 T cells by SEC1 is APC-restricted and TCR Vβ-dependent. CD8+CD25- T cells were purified from human PBMCs and stimulated with SEC1 (1 ng/ml) in the presence or absence of CD14+ cells.

(A) After 6 days stimulation, expression of CD25 and FOXP3 was measured using flow cytometry. Data shown are representative results from nine independent experiments measured from three different donors. (B) After stimulation with SEC1 (1 ng/ml) for 10 min, cells were lyzed and cell lysates were analyzed by immunoblotting using mAb specific to phosphorylated CD3ζ as an indication of T cell activation via TCR. The level of β-actin was used as loading controls. (C) After 4 days stimulation with SEC1 (1 ng/ml), the selective expansion of TCR Vβ was measured by quantitative real time PCR. Data shown are the mean ± SEM of six independent measurements from two donors. (D) Expression of TCR Vβ14 in CD8+CD25+FOXP3+ T cells was measured by flow cytometry. Data shown are the mean percentage of CD8+CD25+Vβ14+ T cells combined from six independent measurements from two donors. (E) CD8+CD25+ T cells were induced by stimulation of purified CD8+CD25- T cells with SEC (1ng/ml) in the presence of CD14+ cells. Purified CD8+CD25+ T cells were co-cultured as described in Fig. 3. Data shown are a single representative CFSE dilution histograms from nine independent experiments measured from three different donors.

Although it is well documented that CD4+ T cells are activated by SAgs that directly bind to specific Vβ segments of TCR on CD4+ T cells and MHC II on APC [188], the exact mechanism by which CD8+ T cells are activated by SAgs is not clearly

understood. For instance, it is possible that induction of CD8+CD25+FOXP3 T cells by SEC1 might be due to a direct activation of CD8+ T cells by SEC1 or by an indirect effect following activation of CD4+ T cells by SEC1. To address these questions, naïve CD8+CD25- T cells were purified from human PBMCs and stimulated with SEC1 at the suboptimal stimulation condition in the presence or absence of APC (CD14+ cells). In the presence of APC, SEC1 induced expression of CD25 and FOXP3 in CD8+ T cells (Fig. 2.9A). In contrast, in the absence of APC, SEC1 was unable to induce expression of CD25 and FOXP3 in CD8+ T cells (Fig. 2.9A). These results indicated that activation of CD8+ T cells by SEC1 requires the presence of APC. To determine whether activation of CD8+ T cells by SEC1 is initiated through the TCR signaling pathway, CD8+ T cells were incubated with SEC1 for 10 min and the phosphorylation of ITAMs in the CD3ζ chain, the initial event in the TCR signaling cascade, was measured by Western blot. The phosphorylation of the CD3ζ chain was detected from CD8+ T cells activated with SEC1 in the presence of APC, not in the absence of APC (Fig. 2.9B). To determine whether activation of CD8+ T cells by SEC1 is also TCR Vβ-dependent, the distribution of the TCR Vβ repertoire in CD8+ T cells before and after stimulation with SEC1 was measured using quantitative real time PCR. Results showed that selective expansion of CD8+ T cells bearing TCR Vβ 3, 12, 13.2, 14, 15, 17, and 20 occurred (Fig. 2.9C). These results were identical to those previously observed with CD4+ T cells [148]. Flow cytometry results further confirmed the quantitative real time PCR results showing an increase of Vβ 14 after stimulation with SEC1 (Fig. 2.9D). Lastly, CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 in the absence of CD4+ T cells were able to suppress proliferation of responder cells (Fig. 2.9E), indicating induction of CD8+

Tregs by suboptimal stimulation with SEC1 is independent from CD4+ T cells. Together, these results suggest that SEC1 directly activates CD8+ T cells via the TCR signaling pathway in an APC restricted- and TCR Vβ-dependent manner, similar to the mechanism documented in CD4+ T cells. The results also demonstrate that suboptimal stimulation induces expansion of immunosuppressive CD8+CD25+FOXP3+ T cells.

2.5 Discussion

It has long been postulated that staphylococcal SAgs contribute to the pathogenesis of *S. aureus* since they are potent immune modulators. However, while toxic shock syndrome caused by exposure to high concentrations of SAgs (1-100 μg) are well documented, relatively little is known about the effect of low concentrations of SAgs (1-10 ng) associated with more frequent types of asymptomatic mucosal colonization or chronic infections by *S. aureus*. A recent study demonstrated that stimulation of human PBMCs with SAgs at a concentration of 1 ng/ml induced immunosuppressive CD8+CD25+FOXP3+ T cells [119]. However, the mechanism of suppression still remains elusive and the functional characteristics of CD8+CD25+FOXP3 induced by stimulation with a higher concentration of SAgs have not been tested. In this study, we demonstrated, for the first time to our knowledge, that the concentration of SAgs markedly influences the functional characteristics of CD8+ T cells. Specifically, suboptimal stimulation by SAg induces functionally immunosuppressive CD8+ regulatory T cells, whereas an optimal stimulation by SAg induces expression of phenotypic markers of CD8+ regulatory T cells, but these cells are not functionally immunosuppressive. We further determined that immunosuppression by CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 was mediated primarily by galectin-1

and granzymes in a contact-dependent and soluble factor-dependent manner, respectively. Our results suggest that exposure to a higher concentration of SAg induces T cell activation without development of Tregs leading to uncontrolled inflammation, likely contributing to conditions such as toxic shock syndrome. Our results further suggest that exposure to lower concentrations of SAg induces T cell activation leading to development of Tregs, resulting in immune suppression. Together these results demonstrate that SAgs can induce completely opposite immune responses (i.e., toxic shock syndrome versus immune suppression) depending on the concentration of SAg presented to the host immune system.

A limitation in investigating Tregs in humans is continued identification of, and debate about, specific markers. Although FOXP3 has an essential role in development of Tregs, as demonstrated in this study and others, FOXP3 may not be a reliable marker for Tregs especially in humans since T cell activation via TCR transiently induces expression of FOXP3 in both CD4+ and CD8+ T cells [122, 123]. In line with others' findings, our results showed that CD8+CD25+ T cells induced by optimal stimulation with SEC1 also highly expressed FOXP3 but were not immunosuppressive. Thus, expression of FOXP3 is necessary for the development of Tregs, but not sufficient to define Tregs.

Induction and development of FOXP3+ Tregs require synergistic interaction of cellular signaling pathways. They include suboptimal stimulation through TCR/MHC II without CD28/CD86 costimulatory signals or with inhibitory signals by CTLA-4. These signaling cascades lead to inactivation of PI3K-AKT-mTOR (phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin) pathways, while activation of inhibitory cytokine pathways, such as TGF-β/TGF-β receptor and TNF-α/TNF receptor superfamily

[189-191]. In this study, the phenotypes and cytokines produced by CD8+CD25+FOXP3+ T cells induced by suboptimal stimulation with SEC1 were CD28+, CTLA4+, TNFR2+, CD45RO+, HLA-DR+, IL-2+, IL-10+, IFN-γ+, and TGF-β+, which satisfied several conditions to induce FOXP3+ Tregs, i) suboptimal stimulation through TCR/MHC II, ii) induction of CTLA-4 expression, and iii) induction of TGF-β and IL-10 expression. However, it is noteworthy that the non-immunosuppressive CD8+CD25+FOXP3+ T cells induced from optimal stimulation with SEC1 also showed similar phenotypes and cytokines to those observed with the immunosuppressive CD8+CD25+ T cells induced by suboptimal stimulation. A recent study demonstrated that stimulation of T cells in the presence of IL-2 and TNF receptor superfamily ligands (GITR-L, OX40-L, 4-1BB-L, and TNF) induced higher expression of FOXP3 than stimulation without those ligands. This would explain higher expression of FOXP3 in CD8+CD25+ T cells induced by optimal stimulation in which GITR and OX40, and TNFR2 were more highly expressed than with suboptimal stimulation. However, functional maturation of Treg may require additional signals. The CD28 molecule, highly expressed in CD8+CD25+ T cells induced by both suboptimal and optimal stimulation conditions, may contribute to the functional maturation of FOXP3+ T cells differently. Previous studies demonstrated that a co-stimulation signal through CD28 is required for tTreg development by inducing expression of TNF receptor superfamily members. However, strong co-stimulation through CD28 suppressed the differentiation of pTreg or iTreg. Importantly, a recent study demonstrated that SAg can directly interact with CD28, which is essential to elicit an inflammatory cytokine storm in toxic shock syndrome [192]. It is possible that the excessive amount of SAg in optimal

stimulation conditions may directly interact with CD28 and induce strong co-stimulatory signals that differentially activates TNF receptor superfamily signaling pathways leading to CD8+CD25+FOXP3+ T cells without a suppressive activity. Further study is necessary to determine the role of SAg interaction with CD28 in the functional maturation of CD8+ Tregs.

A number of studies have reported diverse mechanisms of immunosuppression by CD8+ Treg. The CD8+ Tregs originally reported in the murine model interacted with activated CD4+ T cells expressing the MHC class 1b molecule, Qa-1, via TCR and suppressed self-reactive autologous CD4+ T cells [193]. Clinical studies in humans revealed that human CD8+ Tregs interacted with the HLA-E, equivalent to murine Qa-1, expressed on self-reactive CD4+ T cells and suppressed self-reactive CD4+ T cells by killing antigen-activated autologous CD4+ T cells in autoimmune type 1 diabetes and multiple sclerosis [194]. Other types of CD8+ Tregs suppressed immune responses by mediating negative signals to APC via cell-to-cell interaction such as CTLA-4 and CD80/86 and immunoglobulin-like transcript (ILT) 3 and ILT4 on APC [117], inducing immunomodulatory cytokines such as TGF-β and/or IL-10, and altering metabolic activities such as inducing cytotoxic tryptophan catabolism through induction of indoleamine 2,3-dioxygenase (IDO) [114]. We determined the role of CTLA-4, IFN-γ, IL-10, TGF-β, cAMP, and COX previously described to mediate immunosuppression by Tregs. However, none of these molecules were responsible for immunosuppression by CD8+CD25+ T cells induced by suboptimal stimulation with SEC1.

In a search for unique phenotypic and functional markers of CD8+ Treg, we found that galenctin-1 and granzyme A and B were consistently upregulated in

CD8+CD25+ T cells from our RNA-seq and proteomics analyses. Galectin-1, a member of β-galactoside-binding proteins, is expressed by thymic epithelial cells, activated CD8+ T cells, and macrophages and is secreted by a non-canonical mechanism that bypasses the endoplasmic reticulum and the Golgi apparatus [195]. Galectin-1 interacts with CD4, CD7, CD43, CD45, and CD69 and extracellular matrix glycoproteins such as laminin, fibronectin, and vitronectin and regulates cell homeostasis and inflammation [196]. Previous studies demonstrated that addition of galectin-1 to activated human T cells and leukemic cell lines triggers the clustering and polarization of CD7, CD43, and CD45, followed by signaling events leading to apoptosis [195]. A recent study demonstrated that galectin-1 is highly expressed and secreted in activated CD4+CD25+ Treg and the blockade of galectin-1 by anti-Gal-1 mAb significantly reduced the suppressive activity of CD4+CD25+ Treg, suggesting that galectin-1 is a key effector molecule for immune regulation [72]. Granzymes are serine proteases that induce apoptosis of target cells by activating a caspase cascade. Several studies have demonstrated that CD4+CD25+ Treg also express granzyme A and/or B and induce apoptosis in various autologous immune cells in a perforin-dependent but FasL independent manner [197]. In our study, the blockade of galectin-1 and granzymes using an anti-Gal-1 mAb and DCI significantly, but not completely, inhibited the suppressive activity of CD8+CD25+ T cells induced from suboptimal stimulation with SEC1. These results indicate that while galectin-1 and granzymes are mainly responsible for the suppression, there are additional mechanism of suppression. Combined results from autologous MLR using mitomycin C-treated CD8+CD25+ T cells and transwells with anti-Gal-1 mAb strongly suggest that contact dependent suppression is mediated by galectin-1. However, it is noteworthy that

expression of galectin-1 was only detected intracellularly and that galectin-1 was also highly expressed in CD8+CD25+ T cells induced by optimal stimulation with SEC1. These results raise questions regarding the role of galectin-1 in immune suppression by CD8+CD25+ T cells induced by optimal stimulation with SEC1. It is possible that the anti-Gal-1 mAb recognizes an epitope involved in binding to its receptor so that anti-Gal-1 mAb is able to neutralize the binding of galectin-1 to receptors, but unable to detect galectin-1 bound to the receptors on the surface of CD8+CD25+ T cells induced by suboptimal and optimal stimulation with SEC1 since the epitope for anti-Gal-1 mAb is occupied with its receptors. It is also possible that CD8+CD25+ T cells induced by suboptimal stimulation with SEC1 express a unique surface protein that interacts, either directly or indirectly, with galectin-1 to mediate the contact-dependent suppression. These possibilities are currently under investigation. Elucidating the galectin-1 receptor and its ligand on the target cells would be critical to identify specific surface markers of Tregs and understand their suppression mechanism.

In conclusion, our results clearly demonstrate the contribution of SAgs expressed at low concentrations in the pathogenesis of *S. aureus* by inducing immunosuppressive CD8+ Tregs that can deteriorate host cellular immunity leading to bacterial colonization, spreading, and eventually to highly lethal invasive infections. Several population genomics studies revealed that both commensal and clinical *S. aureus* strains more frequently harbor 5-6 SAgs genes (*seg, sei, sem, sen, seo*, and/or *seu*) clustered in the enterotoxin gene cluster (egc) [168, 198-201]. These SAgs are intrinsically regulated to be expressed at low concentrations which have a potential to induce Tregs. These results further support the biological fitness of *S. aureus* strains expressing SAgs at low

concentrations in the pathogenic evolution of *S. aureus*. Further study investigating alteration of cellular signaling pathways in a SAg concentration dependent manner and molecular mechanisms of immunosuppression by CD8+ Tregs will warrant to design novel therapeutic strategies against *S. aureus* infection.

CHAPTER III

INDUCTION OF IMMUNOSUPPRESSIVE CD4+CD25+FOXP3+ REGULATORY T CELLS BY SUBOPTIMAL STIMULATION WITH STAPHYLOCOCCAL ENTEROTOXIN C1

3.1 Abstract

Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) produced by *Staphylococcus aureus* are superantigens with a prominent characteristic of inducing massive activation of T cells and proinflammation, leading to toxic shock syndrome. Paradoxically, increasing evidence indicates that superantigens also induce strong immunosuppression by inducing development of regulatory T cells. Here, we demonstrated that suboptimal stimulation of human peripheral blood mononuclear cells (PBMCs) with a low concentration (1 ng/ml) of staphylococcal enterotoxin C1 (SEC1) induced immunosuppressive CD4+CD25+FOXP3+ T cells whereas optimal stimulation with a high concentration (1 μg/ml) of SEC1 induced non-immunosuppressive CD4+CD25+FOXP3+ T cells. The suppression was mainly mediated in a contact-dependent manner via galectin-1. Both optimal and suboptimal stimulations induced activation of Akt and mammalian target of rapamycin (mTOR) pathway, leading to expression of FOXP3. However, expression of the lipid phosphatase and tensin homolog (PTEN) was only highly induced by suboptimal stimulation. These results suggest an

important role of PTEN in functional divergence in CD4+CD25+ T cells in optimal and suboptimal stimulation with superantigen.

3.2 Introduction

Staphylococcus aureus is a Gram-positive bacterium that resides in skins, nasal mucosa, and respiratory tract of 30 to 40 % of healthy adults at any given time and 70 % of people experience *S. aureus* colonization without any symptom in their lifetime [202]. *S. aureus* also opportunistically causes a multitude of diseases from mild soft tissue infections to life threating diseases such as infective endocarditis, sepsis, necrotizing pneumonia, abscess, and toxic shock syndrome (TSS) [1].

Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) are superantigens (SAgs) that bind directly to the variable region of a subset of β-chains of the TCR and to MHC II. These interactions allow a single SAg to activate up to 25 % of T cells in the periphery and to elicits extensive proinflammatory immune responses causing TSS [146, 147]. However, *S. aureus* more frequently colonize skins and mucosa where low amounts of SAg are produced at insufficient to cause TSS [2-4]. The role of such low amounts of SAgs in *S. aureus* pathogenesis has not been fully appreciated.

Regulatory T cells (Tregs) play an important role in maintaining immune homeostasis, preventing autoimmune diseases, and balancing immune responses during infections to prevent excessive tissue damage [7-10]. However, pathogens also develop a strategy to exploit Tregs to establish successful infections by suppressing host immune response. We have recently reported that stimulation of human PBMCs with SEC1 induced CD8+CD25+FOXP3+ T cells with a striking functional difference depending on the strength of stimulation modulated by the concentration of SEC1.

CD8+CD25+FOXP3+ T cells induced by stimulation with 1 ng/ml of SEC1 showed strong contact-dependent suppressive activity which was largely mediated by galectin-1. By contrast, CD8+CD25+FOXP3+ T cells induced by stimulation with 1 μg/ml of SEC1 were not immunosuppressive. Similar to these findings, other studies also demonstrated the induction of immunosuppressive Treg by suboptimal stimulation with low dose antigens [47, 50]. However, the molecular mechanism by which suboptimal stimulation of T cell induce development of Treg has not been fully understood.

In this study, we stimulated human PBMCs with SEC1 at the dose inducing maximal and a half maximal T cell proliferation as optimal and suboptimal stimulation conditions, respectively. We determined the phenotypic and functional characteristics of CD4+CD25+ T cells induced by stimulation with SEC1. We further compared differential induction of cellular signaling pathways involved in T cell activation, differentiation, and maturation to understand the mechanism of functional divergence depending on the strength of T cell activation modulated by the concentration of superantigen.

3.3 Materials and methods

3.3.1 PBMC isolation

Blood samples were collected from healthy donors after informed consent had been obtained, in accordance with the protocol (13-191) reviewed and approved by the Institutional Review Board at Mississippi State University. Blood was obtained by venipuncture, and peripheral blood mononuclear cells (PBMCs) were prepared from EDTA-treated whole blood by gradient centrifugation using Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO). The isolated PBMCs were resuspended in RPMI 1640 medium

containing 10% heat-inactivated FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

3.3.2 Purification of SEC1

Dr. Scott Minnich (University of Idaho) kindly provided staphylococcal enterotoxin C1 (SEC1) which was purified from *S. aureus* RN4220 (pMIN121) using preparative isoelectric focusing with a broad isoelectric point (3 to 10) and a narrow isoelectric point (6 to 8) range of ampholytes in succession [159].

3.3.3 Cell stimulation

To determine the concentration of SEC1 inducing maximal and a half maximal T cell proliferation to set optimal and suboptimal stimulation condition, respectively, PBMCs were stimulated with various concentrations of SEC1 (0.001-10,000 ng/ml) in 96-well plate at 5×10^5 cells per well and incubated at 37 ℃ in 5% CO_2 for 3 days. Cells were pulsed with 1 μCi of [^{3}H]-thymidine and cultured for additional 18 hours. Cellular DNA was harvested on glass fiber filters, followed by quantification of [^{3}H]-thymidine incorporation measured by liquid scintillation counting.

To prevent potential expansion of preexisting regulatory T cells, CD25+ cells were depleted from PBMCs using CD25 microbead (Miltenyi Biotec, San Diego, CA). The PBMCs depleted of CD25+ cells (PBMC/CD25-) were adjusted to 1.5×10^6 cells per well in 6-well plate. For optimal and suboptimal stimulation, PBMC/CD25- was stimulated with SEC1 at the 1 μg/ml and 1 ng/ml, respectively, for the indicated times.

3.3.4 Immunophenotyping and flow cytometry

For phenotypic analysis of CD4+CD25+ T cells induced from SEC1 stimulation, mAbs specific to human CD4-FITC, CD25-PE/Cy7, IL-2-PE, IL-10-PE and CD45RO-PE were purchased from BD Biosciences (San Jose, CA). The mAbs specific to human FOXP3-allophycocyanin, CD28-PE, CD127-PE, HLA-DR-PE, OX40-PE, GITR-PE, and CTLA-4-PE were purchased from eBioscience (San Diego, CA). anti-TGF-β-PE was purchased from R&D systems (Minneapolis, MN). Anti-human CD120b-PE (TNFR2) was purchased from BioLegend (San Diego, CA). Anti-human galectin-1-AlexaFluor647 was purchased from Santa Cruz Biotechnology (Dallas, TX).

For surface staining, cells were harvested, washed with FACS buffer (eBioscience) and stained for 30 min at 4℃ with CD4-FITC, CD25-PE/Cy7, CD28-PE, CD45RO-PE, CD127-PE, HLA-DR-PE, OX40-PE, GITR-PE, TNFR2, TCR Vβ2-PE, or TCR Vβ14-PE. For enhanced detection of intracellular cytokines, brefeldin A (eBioscience) was added to stimulated PBMC culture 2 h before harvesting. Following surface staining, cells were fixed and permeabilized with the FOXP3/transcription factor staining buffer set (eBioscience). Fixed/permeabilized cells were stained with FOXP3-allophycocyanin, IL-2-PE, IL-10-PE, TGF-β1-PE, galectin-1-AlexaFluor647 or CTLA-4-PE for 30 min at room temperature.

After further washing, stained cells were acquired using a FACSAria III or a FACSCalibur flow cytometers equipped with FACSdiva or CellQuest software, respectively (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data was analyzed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one (FMO) controls or isotype controls were used.

3.3.5 Suppression assays

For suppression assays, CD4+CD25+ T cells and CD4+CD25- T cells were purified from optimal and suboptimal stimulation of PBMC/CD25- cells with SEC1 by negative selection using a human CD4+ Isolation kit (Miltenyi Biotec, Auburn, CA), followed by human CD25 microbeads II kit (Miltenyi Biotec) according to the manufacturer's instruction. These cells were further labeled using a CellTrace FarRed kit (Invitrogen) to separate Treg from responder cells in the autologous mixed leukocyte reaction (MLR).

Autologous naïve CD4+CD25- T cells were purified from naive PBMC/CD25- using a human CD4+ isolation kit (Miltenyi Biotec). These cells were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, eBioscience) to track the proliferation response to anti-CD3/CD28 Dynabeads (Life technologies, Carlsbad, CA). The purity of isolated cells was consistently 95-99 % as determined by flow cytometry analysis (data not shown).

For the suppression assay, FarRed-labeled SEC1-stimulated CD4+CD25+ T cells (1×10^5, 5×10^4) were mixed with CFSE-labeled naïve CD4+CD25- T cells (1×10^5 cells per well, responder cells) in round-bottom 96-well plate. Cells were stimulated with anti-CD3/CD28 Dynabeads at a 1:1 bead to naïve T cell ratio. After 4 days, proliferation of CFSE-labeled CD4+ T cells was determined as the dilution of CFSE signal using a Novocyte flow cytometer (Acea Biosciences). For the contact-dependent suppression assay, SEC1-stimulated CD4+CD25+ T cells were treated with 10 μM of mitomycin C (Sigma-Aldrich) for 2 hours at 37℃ to prevent them from proliferating. After thorough washing with PBS, cells were co-cultured with naïve CD4+ T cells in the presence of

anti-CD3/CD28 beads. For soluble factor-dependent suppression assay, SEC1-stimulated CD4+CD25+ T cells (1×10^5) were placed in the upper chamber of transwell (0.45 μm pore size, Corning Life Sciences, Tewksbury, MA) and autologous CFSE-labeled naïve CD4+CD25- T cells (1×10^5) were added to the bottom chamber and stimulated with anti-CD3/CD28 Dynabeads for 4 days.

In some experiments, mAbs against IL-10 (R&D systems), TGF-β1 (R&D systems), CTLA-4 (eBioscience), and isotype controls (eBioscience) were added at a final concentration of 10 μg/ml in the culture. Antibody to galectin-1 was added at 0.1 μg/ml in the culture (Santa cruz biotechnology) to the CD4+CD25+ T cell culture and incubated for one hour prior to co-culture with responder cells. Additionally, 25 μM of Indomethacin (COX-inhibitor, Sigma-Aldrich), 10 μM of ZM 241385 (Adenosine 2A antagonist, Tocris, Minneapolis, MN), or 50 μM of 3,4-dichloroisocumarin (serine protease inhibitor, EMD Millipore, Billerica, MA) were added to determine mechanisms of suppression.

3.3.6 Protein extraction and immunoblot analysis

Total protein was extracted by lysing stimulated PBMCs with RIPA buffer (Fisher), plus protease inhibitor (GE sciences) and phosphatase inhibitor (ThermoFisher). Protein concentration was determined and equal amounts of protein were loaded onto an 8 % or 12.5 % denaturing acrylamide gel. Separated proteins were transferred to PDVF membrane. Immunoblotting was performed using the following mAbs from Cell Signaling Technology: phospho-mTOR (Ser2448), Akt, S6, phospho-S6 (Ser240/244), and PTEN. mAb against phospho-Akt (11E6, Ser473) was purchased from Santa cruz

biotechnology. The loading control was assessed by immunoblot using anti-β-actin Ab (Sigma-Aldrich).

3.3.7 Statistical analysis

Statistical significance among different treatment groups was analyzed with the student *t* test or the one-way analysis of variance (ANOVA), followed by Tukey's HSD for post hoc test using GraphPad Prism software (Version 7.0.3. La Jolla, CA). Differences with p value < 0.01 or 0.001 were considered statistically significant as indicated in each experiment.

3.4 Results

3.4.1 Stimulation of human PBMCs with SEC1 induced FOXP3 expression in CD4+ T cells

To determine SEC1 concentrations that induce maximal and half maximal proliferation of T cells, we measured human T cell proliferation in response to stimulation with a various concentration of SEC1 (0.01 to 10,000 ng/ml). The T cell proliferation was positively related to the concentration of SEC1 and the maximal T cell proliferation was observed at 1 μg/ml whereas half maximal T cell proliferation was observed at 1 ng/ml (Fig. 3.1A). Therefore, we defined the optimal strength stimulation condition as 1 μg/ml, which induces maximal proliferation and the suboptimal strength stimulation condition as 1 ng/ml, which induces half-maximal proliferation, respectively.

To examine the strength of T cell stimulation on expression of FOXP3 in CD4+CD25+ T cells, CD25-depleted human PBMCs were stimulated with optimal (1 μg/ml) and suboptimal (1 ng/ml) concentration of SEC1 for up to 8 days. In optimal stimulation, expression of FOXP3 gradually increased and peaked at day 4, then

decreased thereafter. In suboptimal stimulation, expression of FOXP3 was delayed until day 4, peaked at day 6 and sustained until day 8 (Fig. 3.1B & C).

We confirmed that the expansion of FOXP3-positive T cells from suboptimal stimulation was due to SEC1 by measuring SEC1-specific Vβ sequences of FOXP3+ T cells (Appendix fig. 4).

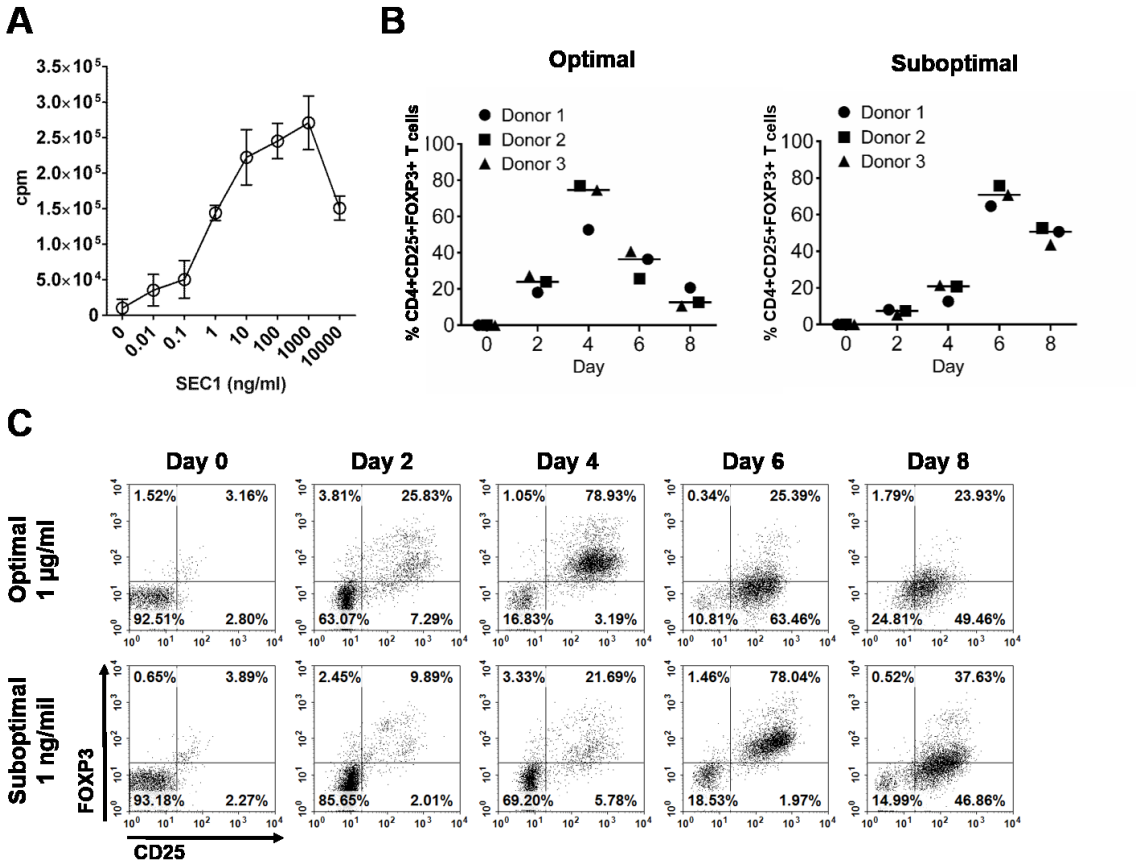

Figure 3.1 FOXP3 expression in CD4+CD25+ T cells stimulated under optimal and suboptimal condition.

(A) Proliferation of human T cells after stimulation with various concentration of SEC1 was measured by the incorporation of ^{3}[H]-thymidine. The data shown are the mean ± SEM of nine independent experiments. (B) Human PBMCs were incubated with optimal and suboptimal condition with SEC1. FOXP3 expression before and after stimulation was measured. Percentage (mean ± SEM) of CD4+CD25+FOXP3+ T cells were obtained with data combined from nine independent experiments. (C) Data shown is a representative of nine independent experiments and gated on CD4+ T cells.

3.4.2 Suboptimal stimulation induces functionally immunosuppressive CD4+CD25+ T cells

As FOXP3 could be transiently expressed in effector CD4+CD25+ T cells, we determine the functionality of CD4+CD25+ T cells induced from optimal and suboptimal stimulation with SEC1 using an autologous MLR assay. As expected, CFSE-labeled naïve CD4+CD25- T cells proliferated well in response to anti-CD3/CD28 beads which was indicated as a gradual dilution of CFSE signal (Fig. 3.2B). CD4+CD25+ T cells induced from suboptimal stimulation with SEC1 completely inhibited proliferation of naïve CD4+CD25- T cells (Fig. 3.2C), whereas CD4+CD25+ T cells induced from optimal stimulation with SEC1 failed to inhibit proliferation (Fig. 3.2D). It is noteworthy that CD4+CD25+ T cells induced from optimal and suboptimal stimulation with SEC1 were labeled with CellTrace FarRed dye and these cells continuously proliferated during the autologous MLR in the presence or absence of naïve CD4+CD25- T cells. Taken together, these results indicated that CD4+CD25+ T cells induced from suboptimal stimulation with SEC1 were functionally immunosuppressive, but cells given optimal stimulation were not functionally immunosuppressive. Suppression was specific to naïve CD4+CD25- T cells, as indicated by the continued proliferation of the SEC1 stimulated cells.

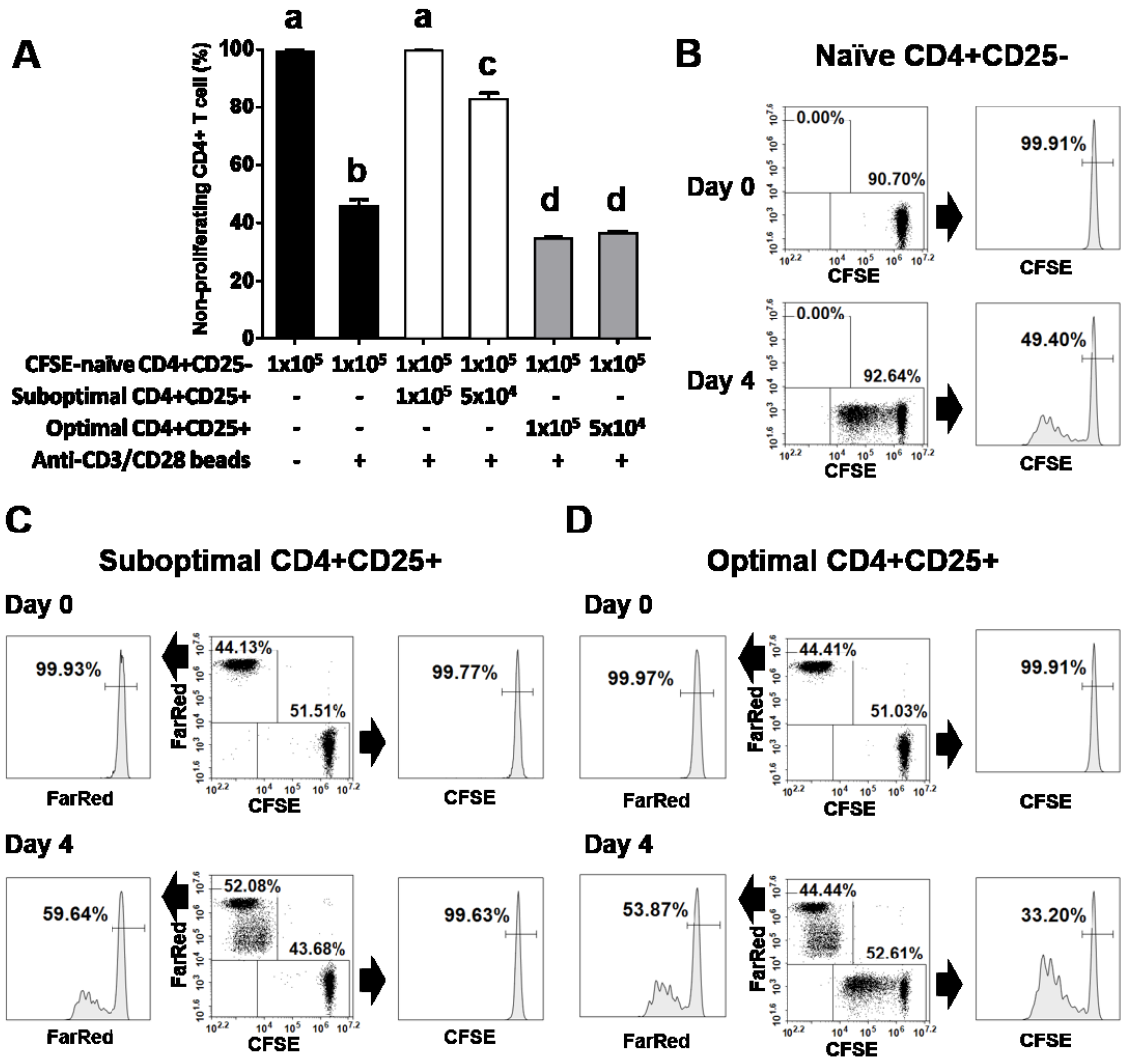

Figure 3.2 CD4+CD25+ T cells induced from suboptimal stimulation are immunosuppressive.

(A) Percentage (mean ± SEM) of non-proliferating responder cells from nine independent experiments. (B) Naïve CD4+CD25- T cells were isolated and stained with CFSE. Proliferation of CD4+ T cells was measured before and after stimulation with anti-CD3/CD28 beads. (C) CD4+CD25+ T cells induced from suboptimal (C) or optimal (D) stimulation with SEC1 were stained with CellTrace Far Red and co-cultured with CFSE-labeled CD4+ T cells at 1:1 ratio in the presence of anti-CD3/CD28 beads. Gating on each cell population is shown in the center dot plot in 3.2C and 3.2D.

Next, we questioned whether the suppression by CD4+CD25+ T cells induced by suboptimal stimulation with SEC1 is mediated by cell-to-cell contact or soluble factors. To examine contact-dependent suppression, CD4+CD25+ T cells induced after

suboptimal stimulation with SEC1 were treated with mitomycin C to prevent de novo T cell activation. The mitomycin C-treated CD4+CD25+ T cells completely inhibited proliferation of naïve CD4+CD25- T cells (Fig. 3.3). However, the suppression by CD4+CD25+ T cells induced by suboptimal stimulation with SEC1 placed in a transwell decreased proliferation of naïve T cells by approximately 40% compared to the contact dependent manner. These results suggest that the suppression by CD4+CD25+ T cells induced from suboptimal stimulation with SEC1 is mainly mediated by cell-to-cell contact, but soluble factors are also involved.

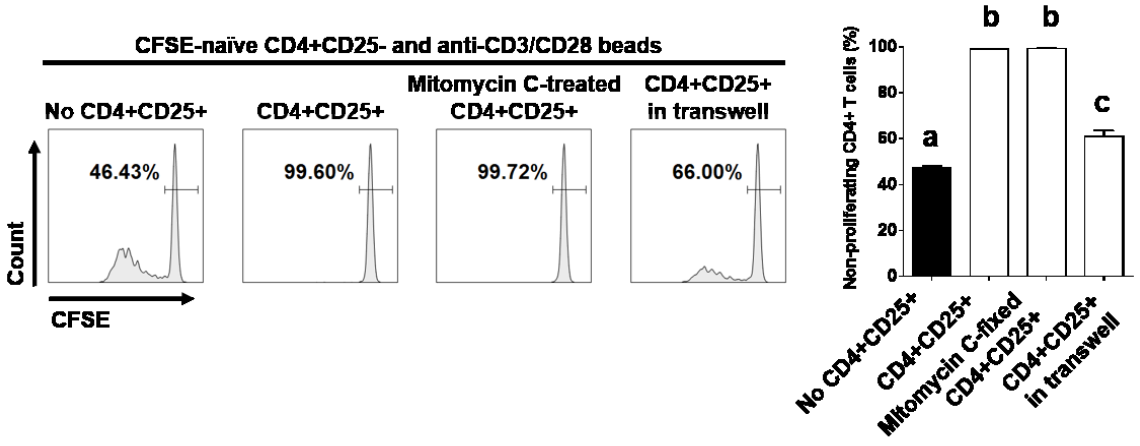

Figure 3.3 Predominant cell-to-cell contact-dependent suppression by CD4+CD25+ T cells induced by suboptimal stimulation.

CD4+CD25+ T cells were isolated from PBMCs after stimulation with a suboptimal concentration of SEC1 and stained with CellTrace Far Red. CD4+CD25+ T cells were treated with mitomycin C for the contact-dependent suppression assay or placed in the top chamber of a transwell for the soluble factor-mediated suppression assay. CFSE-labeled naïve CD4+CD25- T cells were co-cultured as responder cells and stimulated with anti-CD3/CD28 beads. (A) A single representative result is shown. (B) Percentage (mean ± SEM) of non-proliferating CD4+ T cells were obtained by combining results from nine independent experiments.

3.4.3 Expressions of Treg-related surface markers and cytokines in CD4+CD25+FOXP3+ T cells from suboptimal stimulation

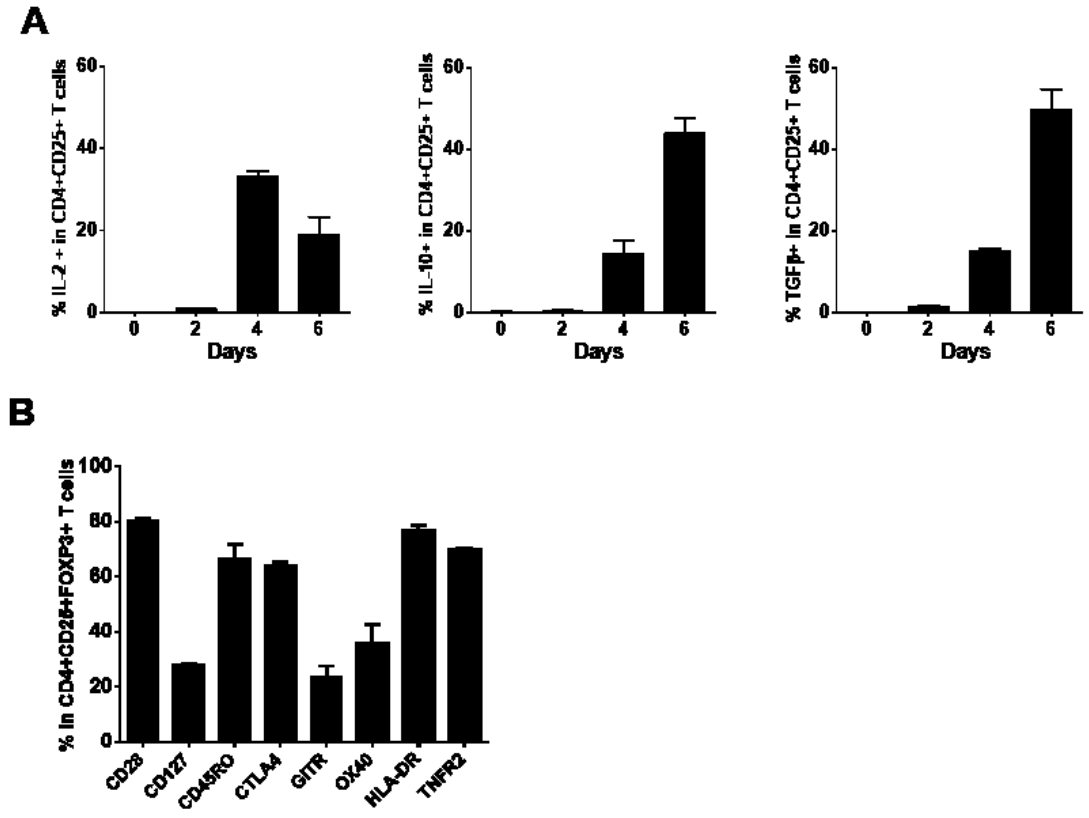

Figure 3.4 CD4+CD25+ T cells induced by suboptimal stimulation express surface markers and cytokines related to Tregs.

Human PBMCs were stimulated with a suboptimal concentration of SEC1 for up to 6 days and expression of cytokines and surface markers related to Treg were analyzed using flow cytometry. Data shown are combined results from nine independent experiments. Cells were treated with brefeldin A for 2 hrs prior to staining and cytokines were measured by intracellular staining. Percentage (mean ± SEM) of CD4+CD25+ T cells expressing cytokines (A) and surface markers related to Tregs (B).

Next, we characterized phenotypes of CD4+CD25+FOXP3+ T cells induced by the suboptimal stimulation with SEC1 using flow cytometry. Upon suboptimal stimulation with SEC1, CD4+CD25+ T cells barely expressed IL-2, IL-10, and TGF-β until day 2. Expression of IL-2 rapidly increased at day 4 then decreased at day 6 (Fig.

3.4A). Expression IL-10 and TGF-β gradually increased throughout the stimulation period. CD4+CD25+FOXP3+ T cells induced by suboptimal stimulation highly expressed CD45RO, CTLA-4, HLA-DR, and TNFR2 and moderately expressed CD127, GITR, and OX40 (Fig. 3.4B). It is noteworthy that expression of CTLA-4 was barely detected on the cell surface but was highly expressed intracellularly. These results are in line with previously described phenotypes of Tregs by us [159] and others [10, 58].

3.4.4 Galectin-1 mediates the contact-dependent suppression by CD4+CD25+FOXP3+ T cells induced by suboptimal stimulation with SEC1

Next, we investigated the mechanisms of suppression by CD4+CD25+FOXP3+ T cells induced by the suboptimal stimulation with SEC1. As CTLA-4, IL-10, and TGF-β are involved in contact-dependent or in soluble factor dependent suppression by Tregs in other models [61, 62, 66, 203], we tested neutralizing Ab against CTLA-4, IL-10, and TGF-β, but failed to restore proliferation of naïve CD4+CD25- T cells (Fig. 3.5A, top panel).

As increased intracellular cAMP caused by activation of adenosine receptor 2A and COX-2 in Tregs suppressed IL-2 production and T cell proliferation [83], we used an adenosine receptor 2A inhibitor (ZM241685) and a COX-2 inhibitor (indomethacin) in autologous MLR. Neither treatment abrogated suppression by CD4+CD25+ T cells induced by suboptimal stimulation with SEC1 (Fig. 3.5A, lower panel).

Since we recently demonstrated the suppression by CD8+CD25+FOXP3+ T cells induced by suboptimal stimulation with SEC1 was mediated by galectin-1 [159], we measured expressions of galectin-1 on CD4+CD25+ T cells induced by suboptimal stimulation with SEC1. Galectin-1 was mainly detected by intracellular labeling and, to a

significantly lesser extent, by surface labeling (Fig. 3.5B). The addition of anti-Gal-1 mAb in the autologous MLR assay partially restored proliferation of naïve CD4+CD25- T cells to approximately 60-65 %, as compared to cells without anti-Gal-1 mAb (Fig. 3.5C & D). The addition of anti-Gal-1 mAb to mitomycin C-treated CD4+CD25+ T cells fully restored proliferation of naïve CD4+CD25- T cells (Fig. 3.5C & D). Taken together, these results strongly suggest that galectin-1 is responsible for contact-dependent suppression by CD4+CD25+ T cells induced by suboptimal stimulation with SEC1.

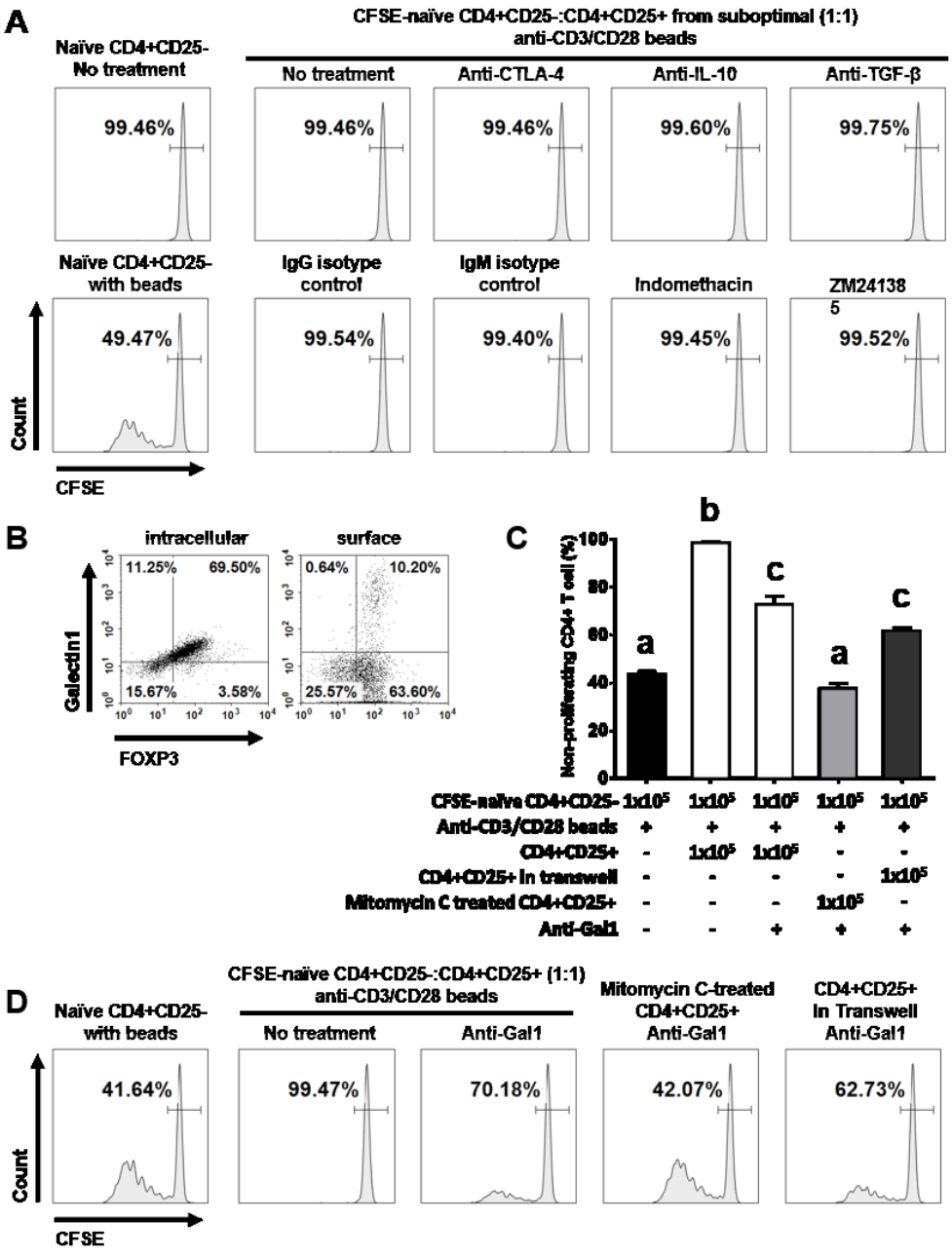

Figure 3.5 Galectin-1 mediates contact-dependent suppression by CD4+CD25+ T cells induced from suboptimal stimulation with SEC1.

(A) CD4+CD25+ T cells from suboptimal stimulation were isolated and co-cultured with CFSE-labeled naïve CD4+CD25- T cells at 1:1 ratio in the presence of anti-CD3/CD28 beads for 4 days. Neutralizing mAb (10 μg/ml) for CTLA-4, IL-10, and TGF-β, indomethacin (25 μM, COX inhibitor), or ZM241385 (10 μM, adenosine 2A antagonist) was added as indicated. (B) Galectin-1 expression in CD4+CD25+FOXP3+ T cells induced by suboptimal stimulation. (C) anti-galectin-1 mAb was added in an MLR reaction with or without mitomycin C treatment of CD4+CD25+ T cells or in transwell. Percentage (mean ± SEM) of non-proliferating CD4+ T cells were obtained from combined results from nine independent experiments. (D) Data shown are a representative dot plots from nine independent experiments.

3.4.5 Differential activation of PI3K-Akt-mTOR pathways depending on the strength of superantigen stimulation

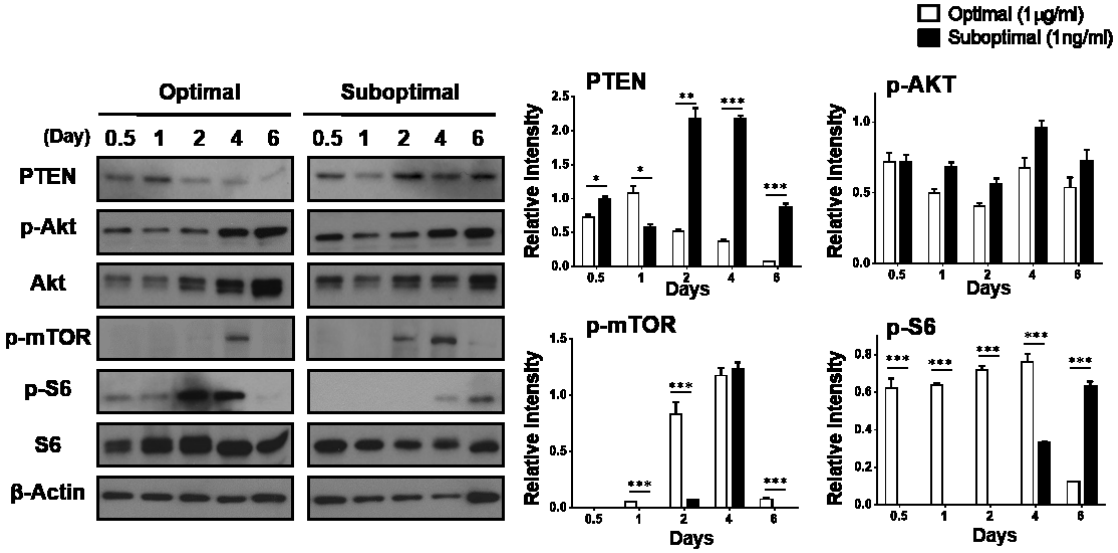

Figure 3.6 Differential activation of PI3K-Akt-mTOR pathway in optimal and suboptimal stimulation with SEC1.

PBMCs were stimulated with optimal or suboptimal concentration of SEC1 for up to 6 days and total protein was extracted at indicated time. Immunoblot analysis of total and/or phosphorylated S6, Akt, mTOR, PTEN, and β-actin. Results shown are a single representative immunoblot data (left). The band intensity was measured using ImageJ software. Relative band intensity was calculated by normalizing the band intensity to its total protein (for S6 and Akt) or β-actin (for mTOR and PTEN). Results combined from three independent experiments were shown (Right). Statistical significance between optimal and suboptimal stimulation was determined by student *t* test (*p<0.01, ** p <0.001, *** p <0.0001).

As the downstream of TCR signaling via PI3K, p110α, p110δ, Akt, and mTOR play an important role in expression of FOXP3 [51, 204], we investigated differential activation of these signaling pathways in CD4+CD25+ T cells induced by optimal and suboptimal stimulation with SEC1 (Fig. 3.6). Expression of PTEN (phosphatase and tensin homolog), which negatively regulates the PI3K activity [205], was slightly increased until day 1 and then gradually decreased thereafter in optimal stimulation. By contrast, expression of PTEN was gradually increased and peaked at day 4, and then decreased thereafter. Phosphorylation of Akt at serine 473 was comparable in both

stimulation conditions and remained constant throughout the stimulation periods. In optimal stimulation, the phosphorylation of mTOR was gradually increased, peaked at day 4 and rapidly decreased thereafter. In suboptimal stimulation, phosphorylation of mTOR spiked at day 4. Upon optimal stimulation, phosphorylation of S6 protein was increased and sustained until day 4. By contrast, the phosphorylation of S6 protein was delayed until day 4 and increased at day 6 in suboptimal stimulation

3.5 Discussion

In this study, we demonstrated that the strength of T cell stimulation modulated by the concentration of superantigen remarkably influences the functional characteristics of CD4+CD25+ T cells. We showed that expression of FOXP3 in CD4+CD25+ T cells was induced by both optimal and suboptimal stimulation of PBMC with SEC1. However, only the CD4+CD25+ T cells induced by suboptimal stimulation are immunosuppressive Tregs. These results suggest that expression of FOXP3 is necessary but not sufficient to the functional maturation of immunosuppressive Treg. The FOXP3 is a transcriptional factor which is essential for induction and maturation of Tregs. However, FOXP3 biology in humans is quite complicated. Although murine FOXP3 is exclusively expressed in CD4+CD25+ Tregs, human FOXP3 can be transiently expressed in effector CD4+ T cells following TCR activation. Only one form of FOXP3 is observed in mice, but at least 4 different alternatively spliced FOXP3 transcripts have been identified in humans (3, 24-26). The human *FOXP3* gene is located on the X chromosome and contains 11 coding exons (exons 1-11) (27). Each exon encodes distinct functional domains comprised of an N-terminal proline-rich domain (a.a. 1-193) responsible for transcriptional repression, a zinc finger (a.a. 200-223), a leucine-zipper-like motif (a.a. 240-261) responsible for

homo-dimer or tetramer formation, and a C-terminal FKH domain (a.a. 388-421) responsible for DNA binding [36]. Since alternative splicing is a highly regulated biological process that generates multiple functional proteins from a single gene, alternatively spliced isoforms of human FOXP3 may have different functions, depending on what functional domains are present. It is of interest to investigate the FOXP3 isoforms present in optimal and suboptimal stimulation which would reveal the specific FOXP3 isoforms leading to functional maturation of suppressive Tregs.

We identified galectin-1 as the effector molecule exerting cell-to-cell contact mediated suppression in Tregs induced by suboptimal stimulation with SEC1. It is noteworthy that SEC1-induced Tregs could suppress responder T cells in the absence of accessory cells such as APCs, indicating direct contacts between CD4+CD25+ T cells and responder T cells for suppressive activity. Galectin-1 is a member of carbohydrate-binding protein family which has emerged as a novel regulator of immune response. The immunoregulation by galectin-1 is conferred via inducing apoptosis of activated T cells [206]. In line with our finding, suppression by galectin-1 was demonstrated in human tTregs by Garin et al. [72]. Although galectins are considered soluble proteins, the presence of surface-bound galectin-1 has been described [72, 207, 208], which supports the potent contact dependent suppression in our study. In SEC1-induced Tregs the majority of galectin-1 resided in the cell while a relatively small portion of galectin-1 was located on the cell surface (Fig.3.5B). It is undetermined how galectin-1 selectively induces apoptosis of activated responder T cells without harming SEC1-induced Tregs. Despite the sharp difference in suppressive function, CD4+CD25+ T cells from optimal and suboptimal stimulation express similar levels of galectin-1 intracellularly and on the

cell surface (Fig. 5B, Appendix fig.5). This indicates the possibility of additional mechanisms which enable galectin-1 to act functionally in Tregs induced by suboptimal stimulation. Galectin-1 is secreted in an ER/Golgi-independent way and is thought to be packaged into intracellular vesicles (exosomes) prior to export . However, it remains elusive how galectin-1 accumulates at the plasma membrane and what triggers vesicle formation. Therefore, TCR signal strength may regulate accumulation of galectin-1 and/or vesicle formation for secretion of galectin-1. It is also possible that galectin-1 binds to specific receptors exclusively expressed on Tregs induced from suboptimal stimulation, which allows galectin-1 to confer suppressive activity by cell-to-cell contact. A recent murine study identified that galectin-9 binds to the extracellular domain of Death Receptor 3 (TNFRSF25) in Tregs, thereby playing a role in expansion and function of Tregs [209]. We are currently investigating binding partners of galectin-1 specifically expressed on cell membrane of Tregs induced from suboptimal stimulation with SEC1.

TCR signal strength, determined by antigen dose, drives T cell fate [47]. Among TCR downstream signaling pathways, the PI3K-Akt-mTOR pathway is specifically considered a gate controller that dictates T cell differentiation into effector T cells or Tregs [51]. Thymus-derived Tregs exhibit diminished Akt phosphorylation compared with effector T cells [204] and abrogation of activation signals in the PI3K-Akt-mTOR pathway is necessary for Treg differentiation [210, 211]. Mammalian/mechanistic target of rapamycin (mTOR) is important for the regulation of T cell activation, differentiation, and function. In general, mTOR in T cells is activated by TCR engagement in the presence of costimulation as well as cytokines. Upon T cell activation via TCR engagement, the PI3K-Akt signaling pathway is induced which activates two distinct

mTOR complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTOR1 activity is typically assessed by the phosphorylation of S6 kinases, while the mTORC2 activity is assessed by the phosphorylation of Akt at serine 473 [212]. The activation of mTORC1 typically enhances protein synthesis and alters metabolism that promotes cell growth, proliferation, and survival. The activation of mTORC2 also enhances cell survival, metabolism, and cytoskeletal rearrangement [212]. It has been proposed that the induction of mTORC1 activity in FOXP3+ Treg via TCR-PI3K-Akt signaling activates metabolism of lipid biosynthesis and cholesterol that triggers proliferation of Treg, and induces expression of CTLA-4 and ICOS to mediate suppression by Treg [213]. In contrast, the induction of mTOR1 activity in naïve CD4+ T cells via TCR-PI3K-Akt signaling inhibits expression of FOXP3 and favors the generation of Th1, Th2, and Th17 T cells [210]. In this study, we removed CD25+ T cells from PBMCs to prevent expansion of preexisting tTreg in PBMCs. Thus, our cellular signaling analysis was from naïve CD4+CD25- T cells responding to the superantigen. Interestingly, in both stimulation conditions, the phosphorylation of mTOR and S6 was coincident with or preceding the expression of FOXP3. The phosphorylation of Akt at serine 473 was sustained in both stimulation conditions. These results indicated that expression of FOXP3 was induced while both mTORC1 and mTORC2 activity were induced. These results were also somewhat contradictory to previously proposed roles of mTORC1 in naïve CD4+ T cells described above. As the proposed role of mTOCR1 in tTreg and naïve CD4+ T cells was largely based on murine models, it still remains elusive whether the role of mTORC1 activity in inducing FOXP3 expression is different in humans or unique to superantigens.

In the current study, CD4+CD25+FOXP3+ T cells induced by suboptimal stimulation matured under high expression of PTEN whereas CD4+CD25+FOXP3+ T cells induced from optimal stimulation matured under minimal expression of PTEN. The lipid phosphatase PTEN antagonizes the catalytic action of PI3K. Thus, expression of PTEN inhibits induction of PI3K-Akt-mTOR signaling, thereby promoting development of Treg. Recent studies demonstrated that antigen dose controls PTEN expression and function which leads to Treg differentiation [214] and targeted deletion of PTEN causes instability of Treg, leading to the conversion of Treg into proinflammatory effector cells, so-called ex-Treg [52]. These results suggest that PTEN signaling might be a critical driver of functional divergence in CD4+CD25+FOXP3+ T cells induced by optimal and suboptimal stimulation with superantigen.

Staphylococcal superantigens have been well characterized for their ability to provoke extensive T cell activation and hyperinflammation, leading to fatal TSS which occurs when hosts are exposed to microgram quantities of superantigens [215]. However, our data demonstrated that immunosuppressive Tregs were induced from suboptimal stimulation of T cells with superantigens which more frequently occurs during asymptomatic colonization of *S. aureus* in hosts. The established *in vitro* model will be useful to understand the mechanisms of TSS and immunosuppression, two paradoxical immune responses induced by superantigens.

CHAPTER IV

DIFFERNTIAL EXPRESSION OF FOXP3 ISOFORMS IN HUMAN REGULATORY T CELLS INDUCED BY SUBOPTIMAL STIMULATION WITH STAPHYLOCOCCAL ENTEROTOXIN C1

4.1 Abstract

Forkhead box P3 (FOXP3) is a central transcription factor for development and function of regulatory T cells. However, the role of FOXP3 expressed in human conventional T cells upon activation is not fully understood. Here, we investigated profiles of FOXP3 isoforms in CD4+ and CD8+ T cells stimulated with staphylococcal enterotoxin C1 (SEC1) at the concentrations inducing the suboptimal and the optimal stimulation, resulting in generation of immunosuppressive FOXP3+ T cells and non-suppressive FOXP3+ T cells, respectively. Flow cytometric analysis using FOXP3 antibodies detecting different epitopes revealed differential expression of FOXP3 isoforms in CD4+CD25+ and CD8+CD25+ T cells induced from optimal and suboptimal stimulation. Further analysis revealed that CD4+ and CD8+ regulatory T cells from suboptimal stimulation highly express a FOXP3 isoform lacking exon 2 (ΔE2) and partially lacking exon3 (ΔpE3) preferentially localized to the nucleus, compared to those from optimal stimulation. Lentiviral transduction of FOXP3 isoform (full length, ΔE2, ΔpE3) into Jurkat T cells did not induce strong immunosuppressive function. By contrast, when cultured in the media generated from suboptimal stimulation, transduced Jurkat T

cells became more immunosuppressive, suggesting that soluble factors generated from suboptimal stimulation with SEC1 play an important role in induction of immunosuppressive Tregs. In conclusion, our data demonstrated that superantigen concentration determines distinct expression and subcellular localization of FOXP3 isoforms, thereby promoting development of functional Tregs in *S. aureus* infection.

4.2 Introduction

Regulatory T cells (Tregs) represent a specific subset of T cells that play a crucial role in peripheral tolerance and immune homeostasis through suppression of activated immune cells. Tregs are derived from the thymus or converted from conventional CD4+ or CD8+ T cells by stimulation in the periphery or *in vitro* and they express the transcription factor forkhead box P3 (FOXP3) [29, 58, 216]. FOXP3 has been recognized as a master controller for the development, maintenance, and function of Tregs [21, 121]. Mutations in the *FOXP3* gene result in a fatal recessive immune disorder termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans [27] and scurfy syndrome in mice [26], which indicates the critical role of FOXP3 on Treg function in immunoregulation.

The *FOXP3* gene contains 11 coding exons and encodes a protein consisting of several functional domains including a C2H2 zinc finger, a leucine zipper, and a winged-helix/forkhead DNA binding domain. These domains are engaged in FOXP3 oligomerization [217], DNA binding [218], or interacting with other transcriptional factors such as nuclear factor of activated T cells (NFAT), retinoic acid receptor-related orphan receptors (RORγt and RORα), and IFN regulatory factor 4 (IRF4) [36] because FOXP3 functions as a repressor or an activator of target genes [32, 33].

FOXP3 expression in mice exclusively identifies Tregs with suppressive functionality [20, 121]. In humans, however, FOXP3 can be ectopically expressed in conventional T cells by stimulation via TCR with [119, 219] or without conferring suppressive capability [122, 123]. Although FOXP3 is still considered an indispensable regulator for suppressive Tregs in humans, the discrepancy in expression and functionality of FOXP3 has not been fully elucidated. Furthermore, previous studies described the importance of antigen concentration on the induction of Tregs from conventional T cells, which determines the strength of signals delivered via T cell receptor/CD28 co-stimulatory receptor [47, 50]. However, it is unclear how the strength of T cell stimulation regulates induction of functional FOXP3, eventually resulting in suppressive properties of Tregs.

An additional distinct difference between murine and human FOXP3 is the presence of alternatively spliced FOXP3 isoforms in humans which have been identified as a full-length, a variant lacking exon 2 (ΔE2), a variant lacking exon 7 (ΔE7), and a variant lacking both exon 2 and 7 (ΔE2ΔE7) [30, 44, 124]. Transfection of naive CD4+ T cells with FOXP3 full-length or ΔE2 resulted in conflicting functional characteristics with induction of immunosuppressive Tregs [45] or failure to induce functional Tregs [29], respectively. It remains elusive how FOXP3 isoforms are induced and what is their role in development and function of Tregs.

In our previous studies, stimulation of PBMCs with different concentration of superantigens induced FOXP3 expressions, but only CD4+CD25+FOXP3+ and CD8+CD25+FOXP3+ T cells induced from suboptimal stimulation were suppressive whereas the cells from optimal stimulation were not [159]. To investigate molecular

mechanisms underlying functional heterogeneity of FOXP3-expressing T cells induced from stimulation with staphylococcus superantigen, we characterized subcellular localization and profiles of FOXP3 isoforms, followed by evaluating functionality of FOXP3 isoforms. This study suggests that the strength of T cell stimulation modulates expression of specific FOXP3 isoforms which determine subcellular localization, leading to functional maturation of Tregs.

4.3 Materials and methods

4.3.1 PBMC isolation and stimulation

This study was approved by the Institutional Review Board at Mississippi State University. Blood was obtained by venipuncture from healthy volunteers and informed consent was obtained from the volunteers in accordance with the protocol (13-191). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO) and cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 2mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). The isolated PBMCs were stimulated with staphylococcal enterotoxin C1 (SEC1) at optimal (1 μg/ml) or suboptimal (1 ng/ml) stimulation as previously determined [159].

4.3.2 Immunophenotyping and flow cytometry

To determine differential expression of FOXP3 in cells induced from SEC1 stimulation, mAbs specific to human CD4-FITC, CD8-FITC, CD25-PE were purchased from BD Biosciences (San Diego, CA). mAb specific to human FOXP3-allophycocyanin

(clone PCH101) was purchased from eBioscience (San Diego, CA) and anti-human FOXP3-Alexaflour647 (clone 150D) was obtained from Biolegend (San Diego, CA).

Cell staining for flow cytometry was performed according to the manufacturer's instructions. Briefly, surface staining with anti-CD4, CD8, CD25 antibody was performed for 30 min at 4℃. Following surface staining, cells were fixed and permeabilized with the FOXP3/transcription factor staining buffer set (eBioscience) and stained with anti-FOXP3 antibody for 30 min at room temperature. After further washing, stained cells were acquired using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one (FMO) controls were used to verify positive expression.

4.3.3 Protein extraction and immunoblot analysis

Subcellular fractionations were performed using a nuclear-cytosol fractionation kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo-Fisher, Waltham, MA). Protein concentration was determined and an equal amount of protein was loaded onto 8 % denaturing acrylamide gel. Separated proteins were transferred to a PDVF membrane. The following mAbs were used to detect FOXP3 isoforms: the PCH101 clone (eBioscience) and the 150D clone (eBioscience), followed by HRP-conjugated anti-rabbit IgG (GE Healthcare). The loading control for fractionization was assessed by immunoblot against Erk (Cell Signaling Technology, Danvers, MA) and histone H3 (Sigma-Aldrich), followed by HRP-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare), respectively.

4.3.4 Fluorescence microscopy

For confocal microscopic analysis, cells were stimulated under the indicated condition and stained with mAbs against AlexaFluor-conjugated FOXP3 (PCH101, eBioscience) or AlexaFluor488-conjugated FOXP3 (150D, eBioscience) as described above. Fixed and stained cells were centrifugated onto glass slides at 700 rpm for 4 min and mounted in VECTASHIEILD mounting media with DAPI (Vector Laboratories, Burlingame, CA). Images were obtained using Leica TCS SP8 confocal microscope (Wetzlar, Germany).

4.3.5 Identification of FOXP3 splice variants

Total RNA was isolated using RNeasy kits (Qiagen, Valencia, CA) from cells before and after stimulation with SEC1 for the indicated period and cDNA was synthesized using oligo(dT) primers and SuperScript III (Invitrogen) according to the manufacturer's instructions.

For non-quantitative identification of *FOXP3* splicing variants, PCR was carried out on cDNAs using primers amplifying the *FOXP3* coding DNA sequence as the forward primer was complementary to the region of exon 1 (5'-CCTCTTCTTCCTTGAACCCC-3') and reverse primer was designed at the region of exon 10 (5'-CAAACATGCGTGTGAACCAGTGG-3'). PCR products were cloned into pCR®-blunt II TOPO® Vector (Life Technologies) and subsequently sequenced.

4.3.6 Quantification of FOXP3 splice variants

For quantification of FOXP3 splicing variants over time, quantitative real-time PCR (qRT-PCR) was conducted using specific primer sets listed in Table 4.1. Before

calculating a relative ratio of each isoform for total FOXP3, we measured total FOXP3 using a primer set complementary to exon 5 and the junction of exon 5 and 6. To ensure the isoform specificity, primers for detecting FOXP3 splice variants were designed to span the junctional regions. The accuracy of the primers was checked by cloned vectors containing each FOXP3 spliced sequence as inserts (data now shown).

Table 4.1 Primer sets for qRT-PCR

Primer set	Primer sequences (5'→3')	Source
Total FOXP3	GCTGGCAAATGGTGTCTG	[30]
	TGGCAGTGCTTGAGGAAG	
ΔpE3	ACATTTCATGCACCAGGTGCACCCC	This study
	ATTCCAGGCTGGCCACGTTG	
ΔE2ΔE3	TCGCAGCTGCAGGGATCAACGT	This study
	CTGTCCTTCCTG GGT GCA CTG	
ΔE2	ATCGCAGCTGCAGCTCTC	This study
	GTGGTGTGAGGCTGATCATGG	
ΔE7	CTTCCTCAAGCACTGCCA	This study
	CTTGTCGGATGATGCCTGC	
β-actin	TGGCACCACACCTTCTACAATG	This study
	GTCTCAAACATGATCTGGGTCATC	

The qRT-PCR was performed using Power SYBR green master mix and ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Threshold cycles (Ct) values were normalized to the expression of β-actin and the percentage of splice variants was calculated in relation to total FOXP3.

4.3.7 Plasmid constructs and transfection

FOXP3 splice variants were constructed by overlap extension PCR using MSCV (Murine stem cell virus)-IRES (Internal ribosome entry site)-FOXP3-Thy1.1 (Addgene

plasmid #17443) used as a template with primers listed in Table 4.2 and cloned into lentiviral expression vector, pLenti-puro (Addgene plasmid #39481). Sequence integrity of the cloned FOXP3 isoforms was confirmed by DNA sequencing. HEK293T cells were co-transfected with FOXP3 isoform-containing lentiviral vector, psPAX2 (Addgene plasmid #12260), and pMD2.G (Addgene plasmid #12259) using TransIT-2020 transfection reagent (Mirus Bio, Madison, WI). After 48 h, virus-containing supernatants were harvested, filtered with a 0.45 μm PVDF syringe filter, and subsequently concentrated by centrifugation at 20,000 x g for 90 min.

Table 4.2 Primers for lentiviral constructs

Primer	Primer sequences (5'→3')	Source
FOXP3F_BamHI	ATATGGATCCATGCCCAACCCCAGGCCT	This study
FOXP3R_XhoI	ATATCTCGAGTCAGGGGCCAGGTGTAGGGTT	
FOXP3E2F	CCATCGCAGCTGCAGCTCTCAACGGTGGAT	
FOXP3E2R	CACCGTTGAGAGCTGCAGCTGCGATGGTG	
FOXP3pE3F	ACATTTCATGCACCAGGTGCACCCCCTGGAG	
FOXP3pE3R	CAGGGGGTGCACCTGGTGCATGAAATGTGGC	

4.3.8 Lentiviral transduction and functional assay

Exogenous Foxp3 genes were introduced to Jurkat T cells by infection with lentiviruses at multiplicity of infection (MOI) of 5 (viruses/cell) for 24 h in the presence of 10 μg/ml of polybrene (Sigma). Expression of full-length Foxp3 and its splice variants was measured by flow cytometry. For functional assay, transduced Jurkat T cells were cultured in RPMI media (Normal media) or in the culture supernatant harvested from PBMCs stimulated under suboptimal condition (SEC1 1 ng/ml) for 6 d (conditioned media). The SEC1 remaining in conditioned media was removed using His-tag column (Supplementary figure 1). Jurkat T cells expressing FOXP3 isoforms were stained with

CellTrace FarRed (Invitrogen) and cocultured with CFSE-stained human naïve CD4+CD25- T cells at 1:1 ratio. Anti-CD3/CD28 beads were added as a stimulant. The proliferation of CD4+CD25- T cells was measured on day 4 using flow cytometry.

4.3.9 Statistical analysis

Statistical significance was analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Two-tailed paired *t*-test and unpaired student *t*-test were employed. Statistically significant *p*-value is indicated in each figure description.

4.4 Results

4.4.1 Differential expression of FOXP3 isoforms depending on stimulation strength

In our previous study, we demonstrated that FOXP3 expression was commonly induced by optimal and suboptimal stimulation with SEC1 whereas the acquisition of suppressive properties was exclusively observed in CD8+CD25+ T cells and CD4+CD25+ T cells induced from the suboptimal stimulation condition. Therefore, we hypothesized that FOXP3 proteins expressed by suboptimal and optimal stimulation may have different isoforms, which determine functionality of FOXP3. To identify the presence of FOXP3 isoforms, we measured expression level of FOXP3 isoforms by flow cytometry using two different clones of FOXP3 antibodies: the PCH101 clone which recognizes a common region of FOXP3 across all known isoforms and the 150D clone which specifically recognize an epitope encoded by exon 2. In CD4+CD25+ T cells, the level of FOXP3 expressions detected by the PCH101 clone and the 150D clone showed a significant difference in CD4+CD25+ T cells from suboptimal stimulation (Fig. 4.1A). It is noteworthy that the FOXP3 expression level measured by the 150D clone is

significantly lower than that measured by PCH101, indicating the presence of a FOXP3 isoform lacking the exon 2-encoding region. In contrast, no significant difference in FOXP3 expression measured by the PCH101 and 150D clone antibodies was observed in CD4+CD25+ T cells from optimal stimulation. Similarly, CD8+CD25+ T cells from suboptimal stimulation showed a significant difference between PCH101 and150D clone staining whereas cells from optimal stimulation did not (Fig. 4.1B) These results indicate that functional heterogeneity of FOXP3 expressing CD4+CD25+ and CD8+CD25+ T cells could be due to differential expressions of FOXP3 isoforms.

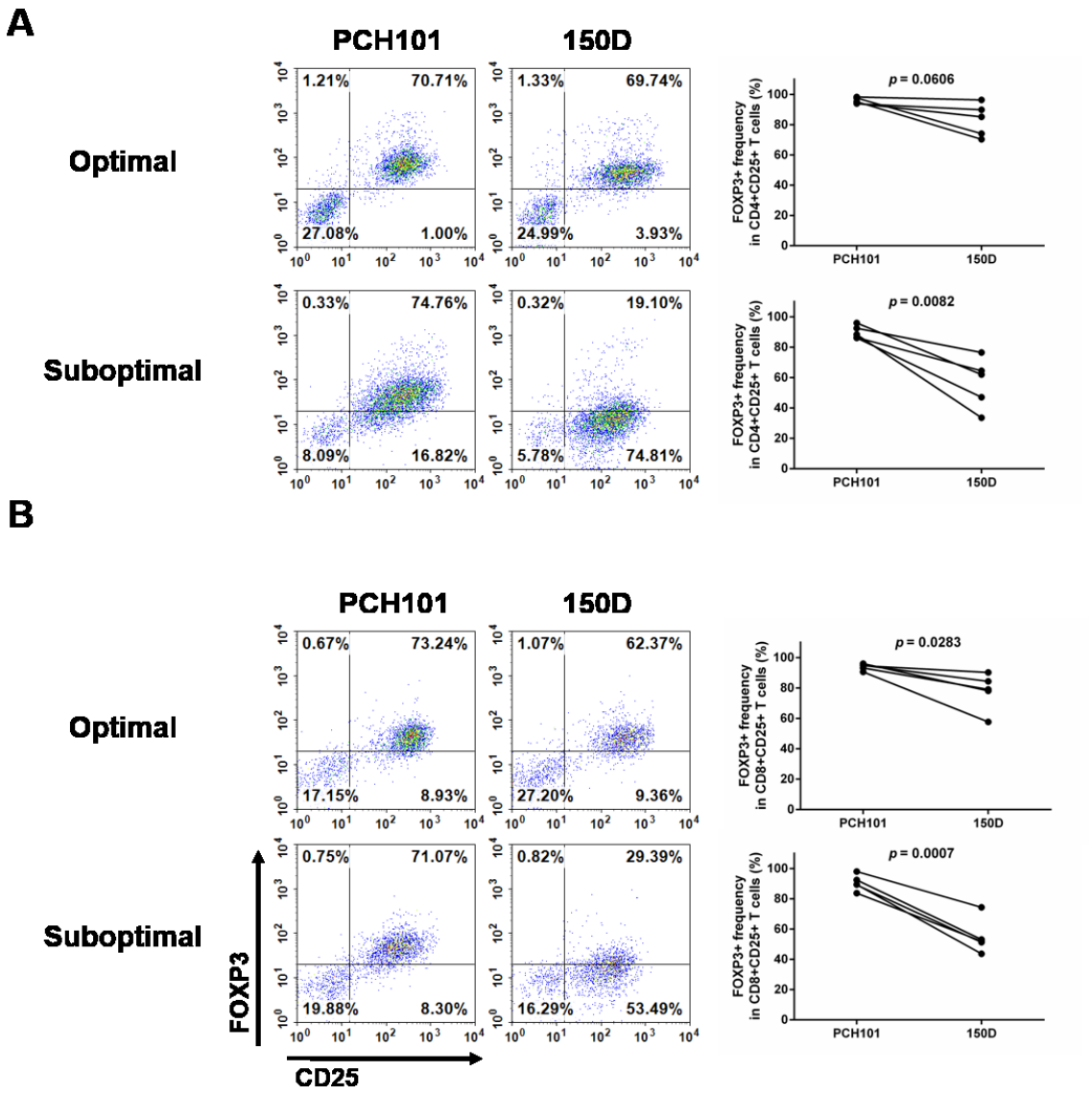

Figure 4.1 FOXP3 isoforms are differentially expressed depending on stimulation strength.

FOXP3 expression was measured by flow cytometry and gated in CD4+ (A) and CD8+ (B) subsets. Each circle in the graph indicates different donors and FOXP3 expression in the same donor is connected by a line. The *p*-value was determined by paired t-test and < 0.01 is considered significant.

4.4.2 Differential subcellular localization of FOXP3 isoforms depending on stimulation strength

Since FOXP3 is a transcriptional factor that binds to DNA to regulate target gene transcription, nuclear localization of FOXP3 is essential to achieve its functionality. Thus, we assessed localization status of FOXP3 under optimal and suboptimal stimulation conditions. Immunoblot analysis using anti-FOXP3 antibody PCH101 clone identified several isoforms of FOXP3 in fractionated protein extracts of PBMCs following stimulation with suboptimal and optimal conditions (Fig. 4.2A). Noticeably, a 49 kDa FOXP3 protein predicted as full-length FOXP3 was observed in the cytoplasmic fraction from both conditions. However, FOXP3 isoforms with molecular weight of approximately 47 kDa and 44 kDa were distinctly observed in the nuclear fraction of suboptimal-induced cells while no band was observed in the nuclear fraction of cells from optimal stimulation. The 44 kDa protein in the cytoplasmic fraction was commonly observed from both stimulation conditions. Next, we performed immunoblotting with the anti-FOXP3 antibody 150D clone which detects an exon 2-encoding region. Interestingly, the band intensity of the 44 kDa-form in the nuclear fraction of suboptimal-induced cells was significantly diminished as compared to the results with the PCH101 clone while the 47 kDa-form was still weakly detected. The 49 kDa and 44 kDa-forms were also detected in cytoplasmic fraction of both cell subsets. Therefore, we assumed that the 49 kDa and 44 kDa proteins in the cytoplasmic fraction and the 47 kDa protein in the nuclear fraction are FOXP3 isoforms containing an exon 2-encoding region whereas the 44 kDa protein in the nuclear fraction is a FOXP3 isoform lacking an exon 2-encoding region.

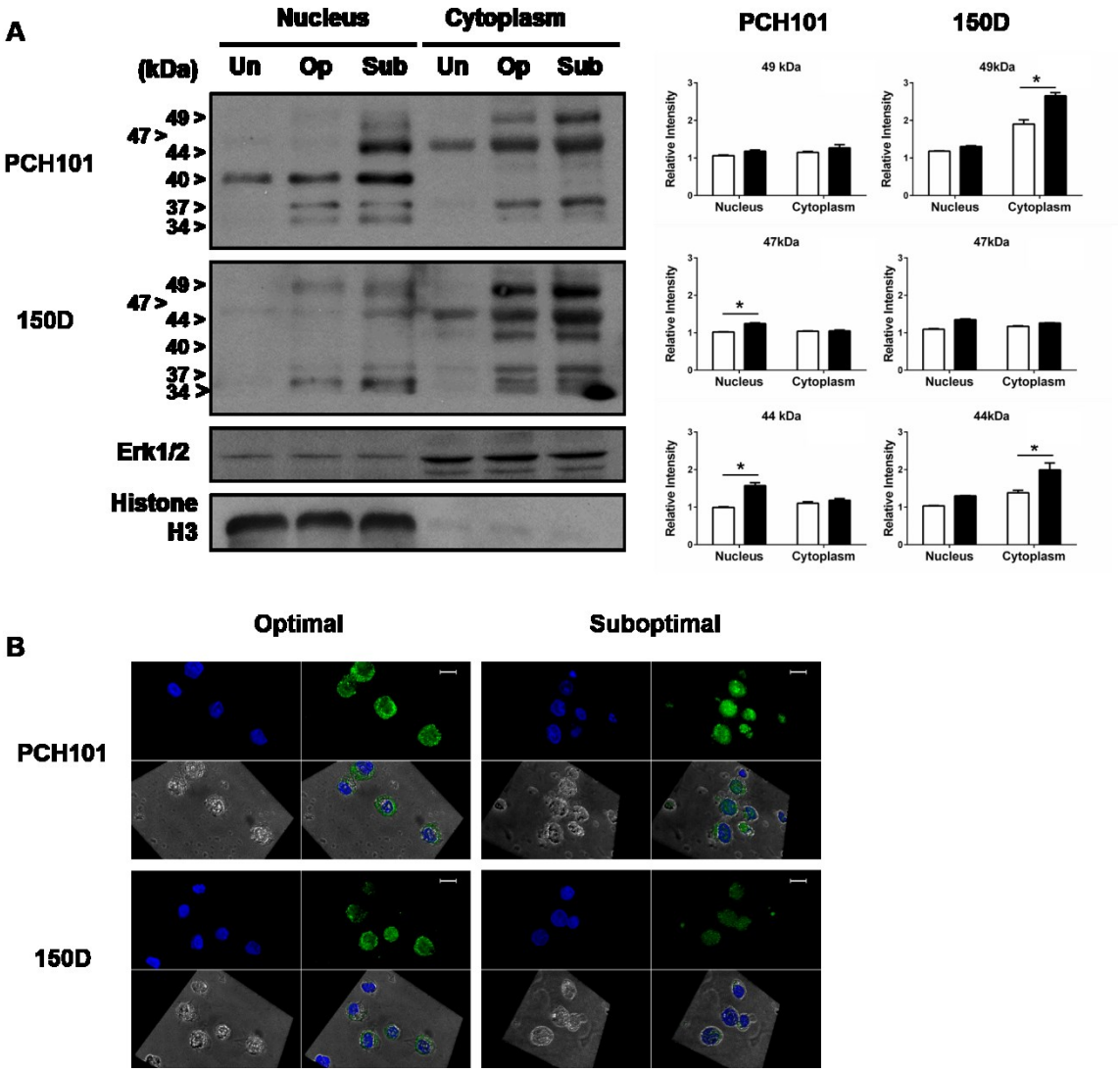

Figure 4.2 FOXP3 isoforms are differentially localized from optimal and suboptimal stimulation.

(A) Immunoblot analysis of FOXP3 and fractionation controls (Erk1/2 and Histone H3) in nuclei and cytoplasmic extracts from stimulated (day 6) or unstimulated (day 0) PBMCs (left panel). Relative band intensity compared to unstimulated condition is shown (right panel). The *p*-value was calculated by student *t*-test and <0.05 was considered significant. (B) Confocal analysis of FOXP3 expression is shown. Cells were stained with FOXP3 mAb clones conjugated with AlexaFlour488 (green) and with DAPI (nuclear stain, blue). Scale bar indicates 1μm.

We further confirmed this result by confocal imaging analysis. Under optimal stimulation, FOXP3 was mostly located in the cytoplasm when stained with the PCH101 and 150D clone antibodies (Fig. 4.2, left panel). In contrast, FOXP3 stained with

PCH101 was localized to the nucleus in cells from suboptimal stimulation (Fig. 4.2, right panel). When stained with the 150D clone, the intensity of FOXP3 signals was significantly reduced, indicating FOXP3 isoforms which lack an exon 2-encoding region are abundant in cells from suboptimal stimulation, corresponding to our results from flow cytometry and immunoblotting.

Taken together, subcellular localization of FOXP3 proteins was diverse depending on the types of isoforms and functionality of cells, indicating the stimulation strength may regulate FOXP3 isoform expressions and its subcellular localization which leads to the functional heterogeneity of FOXP3-expressing T cells.

4.4.3 Novel splicing variants of FOXP3 are induced by superantigen stimulation

To further identify FOXP3 isoforms, we performed amplification of the coding DNA sequence of FOXP3 using cDNA prepared from CD4+CD25+ T cells induced from suboptimal stimulation. The amplification generated several distinct sizes of bands (Fig. 4.3A), which were cloned and subsequently presented for DNA sequencing. The sequencing results showed a library of FOXP3 splice variants, not only previously described isoforms including FOXP3 full-length, FOXP3 lacking exon 2 (ΔE2) and lacking exon 7 (ΔE7), but also novel splice variants including FOXP3 lacking part of exon 3 (ΔpE3) and lacking both exon 2 and 3 (ΔE2ΔE3). Interestingly, FOXP3ΔpE3 partially lacks the first 42 bp of nucleotides in exon 3, different from other splicing variants which lack entire exon sequence as depicted in Fig. 4.3B.

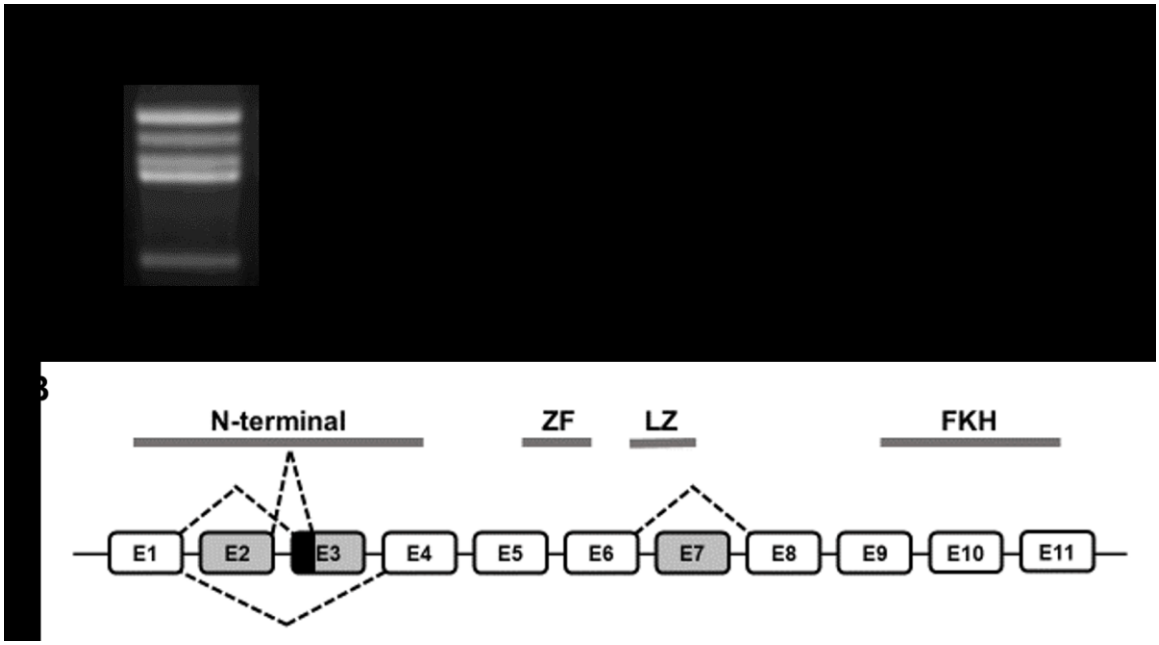

Figure 4.3 Novel splice variants of FOXP3 are induced by stimulation with SEC1.

(A) PCR products from FOXP3 amplification were separated by agarose gel electrophoresis. FOXP3 splicing variants identified by sequencing and size are indicated. (B) Depicted structure of FOXP3 domains and alternative splicing. Dotted line indicates splicing events.

4.4.4 Dynamic changes in expressions of FOXP3 splice variants

To determine whether expression of splice variants is regulated in a time-dependent manner depending on the stimulation strength, mRNAs were prepared from PBMCs at the indicated stimulation period and expression levels of each splice variant were measured by qRT-PCR. Primers were designed specific to each splice variant by spanning splice junctions. To compare the levels of total *FOXP3 e*xpression between stimulation conditions, relative quantification of total *FOXP3* expression was performed using primers recognizing common sequences across splice variants. We observed that the optimal and suboptimal stimulation led to upregulation of *FOXP3* expression at comparable levels during stimulation (Fig. 4.4A). The levels of upregulated *FOXP3* expression on average were similar between stimulation conditions excluding at 48 h post

stimulation when the suboptimal stimulation induced a significantly higher level of FOXP3. Since expression of total *FOXP3* mRNAs was downregulated after 72 h post stimulation (data not shown), subsequent quantification of splice variants was performed for up to 72 h.

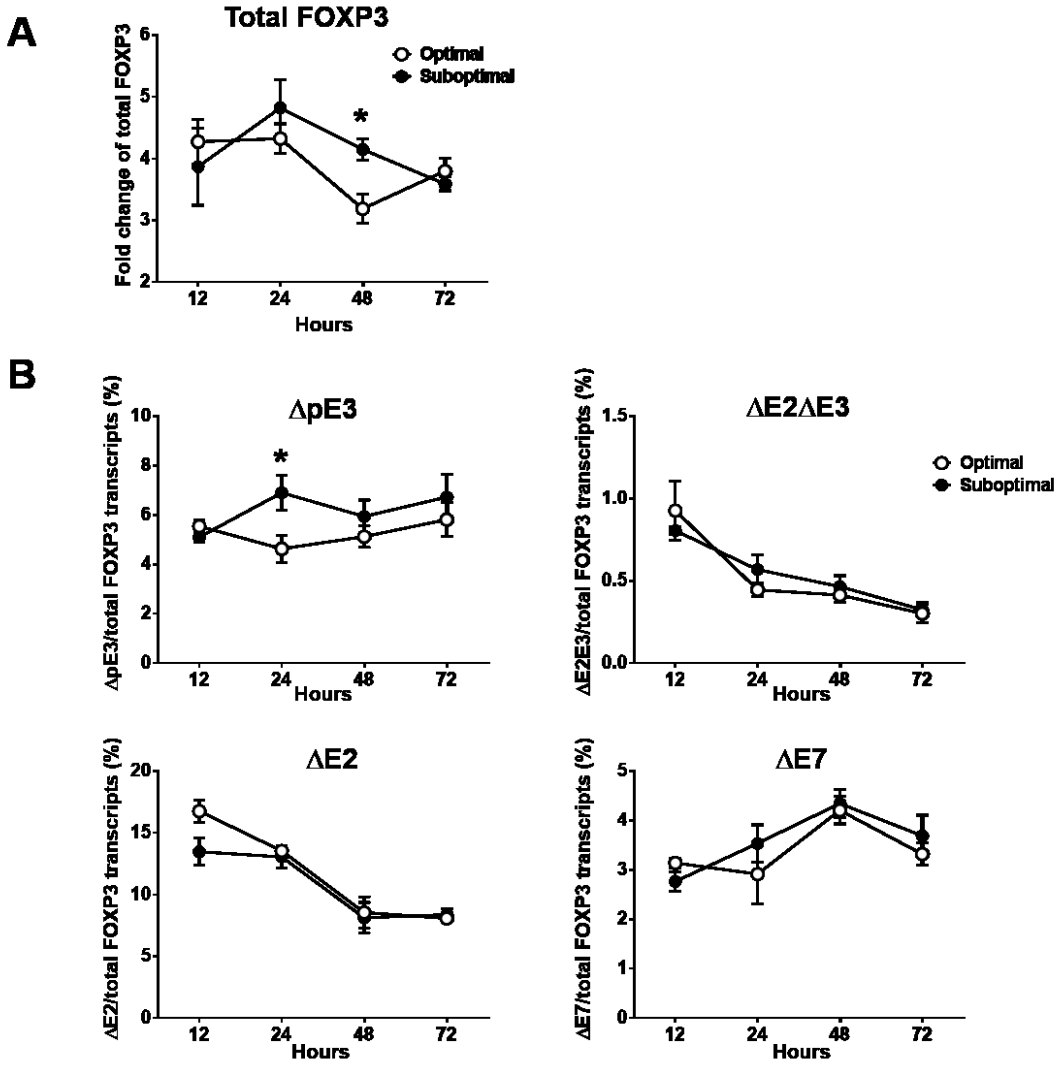

Figure 4.4 Transcriptional change of FOXP3 splice variants.

(A) Total FOXP3 was quantified by qPCR. Data were normalized to β-actin and ddCt was calculated from day 0. (B) Percentage of FOXP3 splice variants in total FOXP3 was measured by qPCR. Data are determined from three independent experiments. Statistical significance was calculated by student t test between stimulation conditions (*$p<0.05$).

Meanwhile, proportions of each splice variant in relation to total FOXP3 transcripts showed active changes following the stimulation period (Fig. 4.4B). During stimulation FOXP3 full-length and ΔE2 were the major splice variants followed by ΔpE3, ΔE7 and ΔE2ΔE3 in both stimulation conditions. Interestingly, the proportion of ΔE2 and ΔE2ΔE3 expressions decreased after 12 h post stimulation while that of ΔpE3 increased. Expression of ΔE7 increased and peaked at 48 h post stimulation, followed by a decrease at 72 h post stimulation. Comparing between stimulation conditions, we observed the proportion of ΔpE3 was significantly higher in suboptimal stimulation than in optimal stimulation at 24 h post stimulation. Overall, expressions of FOXP3 splice variants transcripts changed dynamically throughout the stimulation regardless of stimulation strength.

4.4.5 FOXP3 full-length, ΔE2, and a novel isoform, ΔpE3 confers suppressive functionality which is enhanced by soluble factors produced from suboptimal stimulation with SEC1

Combining results from immunoblot analysis and cDNA sequencing, we assumed that the 47 kDa and 44 kDa proteins detected in nuclear fraction of cells from suboptimal stimulation would be ΔpE3 and ΔE2, respectively, based on protein size prediction. To identify functionality of FOXP3 isoforms, FOXP3 splice variants (full-length, ΔpE3 and ΔE2) were cloned and transduced into Jurkat T cells using lentiviral system (Fig. 4.5A). Subsequently, we performed MLR by mixing transduced Jurkat T cells and naïve CD4+CD25- T cells at 1:1 ratio. Proliferation of naïve CD4+CD25- T cells in response to anti-CD3/CD28 beads was partially inhibited by Jurkat T cells transduced with FOXP3 full-length, ΔpE3, and ΔE2 (Fig. 4.5C). Interestingly, ΔpE3-transduced Jurkat T cells showed stronger suppressive activity compared to FOXP3 full-length and ΔE2-

transduced Jurkat T cells. Furthermore, we tested whether additional signaling affects development of Tregs functionality by culturing transduced Jurkat T cells in conditioned media made from PBMCs with suboptimal stimulation. Interestingly, the suppressive activity was significantly enhanced when transduced Jurkat T cells were incubated with conditioned media (Fig. 4.5D). These results suggest that expression of FOXP3 isoforms including ΔE2 and novel isoform ΔpE3 as well as additional molecules secreted under suboptimal stimulation with SEC1 could be the key factors to induce immunosuppressive Tregs.

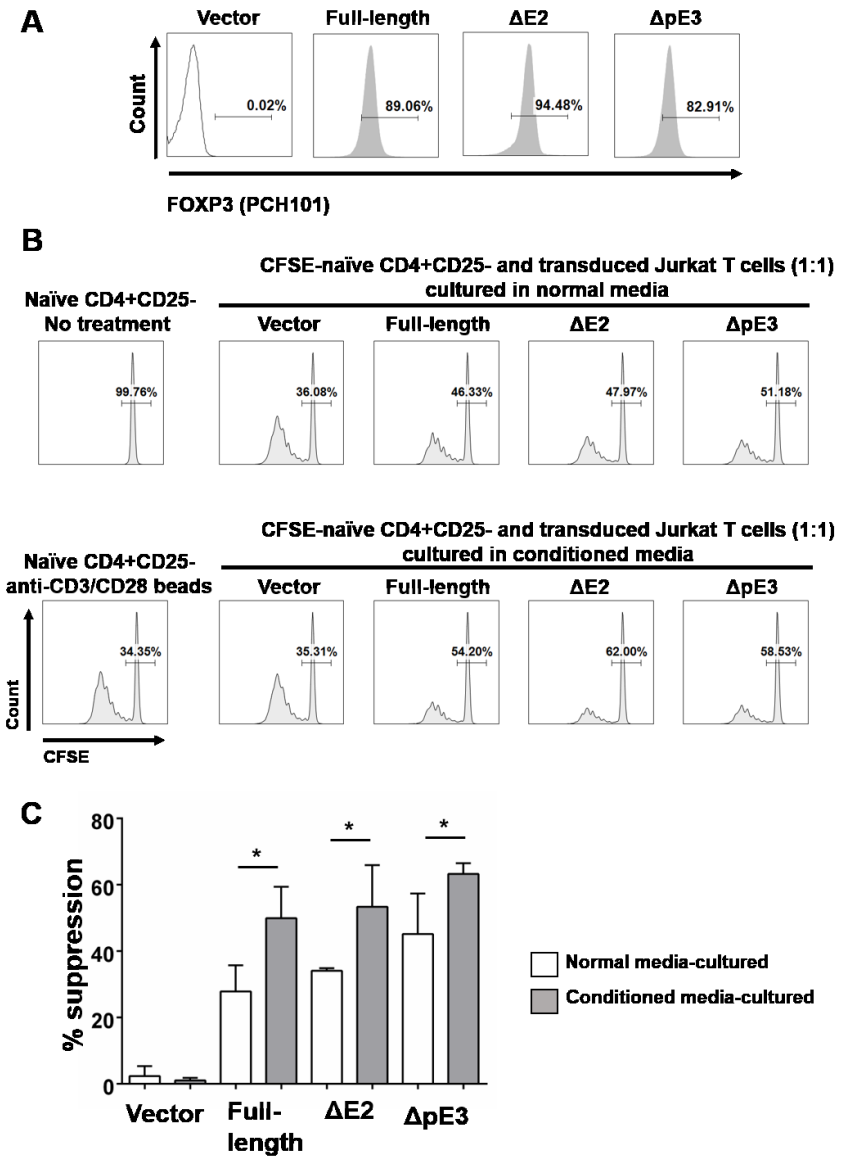

Figure 4.5 Suppression activity conferred by overexpression of FOXP3 full-length, ΔE2, and ΔpE3 was enhanced by soluble factors produced from suboptimal stimulation with SEC1.

(A) Expression of FOXP3 isoforms in Jurkat T cells by lentiviral transduction were measured using flow cytometry. FOXP3 was stained with the PCH101 clone. (B) Transduced Jurkat T cells incubated in normal media or in conditioned media, collected from suboptimal stimulation condition, were stained with CellTrace Far Red and mixed with CFSE-labeled human primary CD4+ T cells in addition of anti-CD3/CD28 beads. Representative figures from three independent experiments are shown. (C) Percentage of relative suppression (mean±SEM) was calculated from three independent experiments. Student *t*-test was performed between culture conditions (*$p<0.05$).

4.5 Discussion

Subcellular localization of FOXP3 serves an important function since FOXP3 binds to DNA directly and regulates expressions of target genes. Previous studies have demonstrated that FOXP3 in activated conventional CD4+ T cells was mostly localized to the cytoplasm while FOXP3 in thymus-derived Tregs was more likely to be localized to the nucleus [42, 220]. In this study, we observed predominant nuclear localization of FOXP3 isoforms with the molecular weights of 47 kDa and 44 kDa in cells from suboptimal condition. Although nuclear import and export signals present in FOXP3 have been studied [41, 42], it is still unclear what regulates FOXP3 localization. It is plausible that FOXP3 localization is regulated by TCR signal strength in line with studies showing that nuclear localization of FOXP3 is enhanced by TCR/CD28 engagement [221] and mTOR inhibitor treatment [220]. It is also possible that other transcription factors are regulated by TCR stimulation strength and subsequently control FOXP3 localization by interaction [43]. Yet, the molecular mechanism how stimulation strength regulates FOXP3 localization needs to be elucidated.

Alternative splicing generally involves exclusion of specific exon(s) as seen in previously identified *FOXP3* splice variants such as *ΔE2*, *ΔE7*, and *ΔE2ΔE7*. Noticeably, *ΔpE3* found in our study employs a distinct alternative spicing event termed alternative 3' splice. When a splice site score of alternative 3' exon was calculated using the Human Splicing Finder as described by Desmet et al. [222], the score (89.95) for the alternative spliced region of exon 3 in *ΔpE3* was higher than the score (88.19) for the intron-exon 3 junction in *FOXP3 full-length*. This indicates that alternative 3' splice is likely to happen in exon 3 and firmly supports our finding. Meanwhile, recent studies showed that T cell

activation leads to altered expression of proteins responsible for immune response via alternative splicing [223, 224]. A recent murine study described that TCR stimulation strength differentially regulates expression of splicing factors, hnRNP L and hnRNP A1 as Akt substrates [225]. Thus, it is possible that TCR stimulation strength determines alternative splicing of FOXP3 and other factors necessary for Tregs differentiation.

The discrepancy in mRNA and protein expression of FOXP3 isoforms may be due to differential stability of FOXP3 proteins regulated by posttranslational modification of FOXP3, specifically polyubiquitination which results in protein degradation. TCR stimulation facilitate FOXP3 polyubiquitination by signaling via Akt-mTOR axis [226] and by shuttling histone deacetylase (HDAC) to the nucleus [227, 228]. Thus, this suggests further studies to identify how stimulation strength regulates stability as well as posttranslational modification status of FOXP3 isoform proteins, which could provide an insight to understand functional heterogeneity of FOXP3-expressing T cells in humans.

From our immunoblot data, we expected that FOXP3 isoforms with molecular weight of 47 kDa and 44 kDa, assumed to be ΔpE3 and ΔE2, respectively, could be the key factors for Tregs function in line with the findings from subjects with relapsing-remitting multiple sclerosis (RRMS) [229] and glycolysis-dependent Tregs induction [230]. It is noteworthy that the novel isoform ΔpE3-transduced cells inhibited proliferation of responder cells as effectively as FOXP3 full-length and ΔE2. These were previously described as major FOXP3 isoforms in humans. The presence of an exon 2-encoding region seems necessary for Tregs functions since increased expression of ΔE2 has been observed in inflammatory diseases with deficient functional Tregs [231, 232]. This is inconsistent with *in vitro* result in this study and others [28, 45] which showed

suppressive activity of ΔE2-overexpressed T cells. Still, compared to potent suppression by Tregs induced from suboptimal stimulation, weak suppression was observed in MLR with FOXP3-transduced cells. This suggests the presence of additional regulators other than FOXP3 to completely induce Treg function. Indeed, the suppression was enhanced by incubation with conditioned media which may contain Tregs differentiation factors. Our results suggest that soluble factors play a role in development of functional Tregs beyond promoting *FOXP3* transcription described in IL-2 and TGF-β [55, 233]. It is also possible that this enhancement was not achieved by a single factor but by a combinatory effect of multiple soluble factors. The responsible factors and molecular mechanisms by which enhance suppressive functionality needs to be elucidated in future studies

In conclusion, our findings demonstrated that the superantigen concentration determines induction of immunosuppressive Tregs via differential expression and localization of FOXP3 isoforms. Given the importance of FOXP3 on acquisition of regulatory functionality, further studies need to focus on a network connecting TCR activation to programming functional FOXP3.

CHAPTER V

CONCLUSIONS

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical diseases from mild skin infections to life-threatening diseases. The objectives of this dissertation research are to determine contribution of staphylococcal superantigens on *S. aureus* pathogenesis and to identify underlying molecular mechanisms of immunomodulation by superantigens. The major findings are concluded as following:

First, a low concentration of superantigen, relevant to more frequent biological conditions in *S. aureus* infection, induced immunosuppressive CD4+CD25+FOXP3+ and CD8+CD25+FOXP3+ Tregs. These results suggest that *S. aureus* may exploit Tregs-mediated immune suppression for its prolonged colonization by secreting a low amount of superantigen and thereby inducing Tregs in local environment. Also, CD8+ Tregs can be induced via direct activation by superantigen and antigen presenting cells, independently from CD4+ T cells. This suggests that superantigens not only activate CD4+ T cells but also CD8+ T cells simultaneously, which leads to an augmented immunomodulatory effect.

Second, CD4+ and CD8+ Tregs induced by suboptimal stimulation with staphylococcal superantigen employed galectin-1 and granzymes for their suppressive activity. Identifying suppression mechanism of Tregs induced from superantigens contributes on understanding of superantigen-induced immunosuppression. This finding

also highlights the role of galectin-1 on immunomodulation and suggests possibility of its therapeutic intervention.

Third, FOXP3 isoforms were differentially expressed and localized by stimulation strength. Since FOXP3 is expressed in human T cells upon activation, the findings in this study firstly identified that stimulation strength controls localization of FOXP3 isoforms and provides insights of discrepancy in FOXP3 expressing cells. Attenuated activation of Akt-mTOR signal pathway was also observed, indicating this pathway is responsible for Treg development in humans aligned to findings in mouse counterparts, although the link between inactivated signals in the pathway and development of functional Tregs remains elusive.

In summary, this dissertation study addresses that superantigens produced under colonization or chronic infection can serve as an immunomodulator for bacterial colonization, propagation, and eruption of highly lethal invasive diseases. The findings in this dissertation provide novel insights into superantigen-mediated immunosuppression which contributes on *S. aureus* pathogenesis and add to our knowledge of superantigens necessary for development of innovative therapeutic strategies against *S. aureus* infection.

REFERENCES

1. Spaulding, A.R., et al., *Staphylococcal and streptococcal superantigen exotoxins.* Clin Microbiol Rev, 2013. **26**(3): p. 422-47.

2. Kulhankova, K., J. King, and W. Salgado-Pabon, *Staphylococcal toxic shock syndrome: superantigen-mediated enhancement of endotoxin shock and adaptive immune suppression.* Immunol Res, 2014. **59**(1-3): p. 182-7.

3. LeClaire, R.D. and S. Bavari, *Human antibodies to bacterial superantigens and their ability to inhibit T-cell activation and lethality.* Antimicrob Agents Chemother, 2001. **45**(2): p. 460-3.

4. Kuroishi, T., et al., *Concentrations and specific antibodies to staphylococcal enterotoxin-C and toxic shock syndrome toxin-1 in bovine mammary gland secretions, and inflammatory response to the intramammary inoculation of these toxins.* J Vet Med Sci, 2003. **65**(8): p. 899-906.

5. Burian, M., et al., *Expression of staphylococcal superantigens during nasal colonization is not sufficient to induce a systemic neutralizing antibody response in humans.* Eur J Clin Microbiol Infect Dis, 2012. **31**(3): p. 251-6.

6. Holtfreter, S., et al., *egc-Encoded superantigens from Staphylococcus aureus are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin.* Infect Immun, 2004. **72**(7): p. 4061-71.

7. Noel, C., et al., *Chronic exposure to superantigen induces regulatory CD4(+) T cells with IL-10-mediated suppressive activity.* Int Immunol, 2001. **13**(4): p. 431-9.

8. Grundstrom, S., et al., *Superantigen-Induced Regulatory T Cells Display Different Suppressive Functions in the Presence or Absence of Natural CD4+CD25+ Regulatory T Cells In Vivo.* The Journal of Immunology, 2003. **170**(10): p. 5008-5017.

9. Mahic, M., et al., *Generation of highly suppressive adaptive CD8(+)CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation.* Eur J Immunol, 2008. **38**(3): p. 640-6.

10. Taylor, A.L. and M.J. Llewelyn, *Superantigen-induced proliferation of human CD4+CD25- T cells is followed by a switch to a functional regulatory phenotype.* J Immunol, 2010. **185**(11): p. 6591-8.

11. Abbas, A.K., et al., *Regulatory T cells: recommendations to simplify the nomenclature.* Nat Immunol, 2013. **14**(4): p. 307-8.

12. Shevach, E.M. and A.M. Thornton, *tTregs, pTregs, and iTregs: similarities and differences.* Immunol Rev, 2014. **259**(1): p. 88-102.

13. DiPaolo, R.J., et al., *CD4+CD25+ T Cells Prevent the Development of Organ-Specific Autoimmune Disease by Inhibiting the Differentiation of Autoreactive Effector T Cells.* The Journal of Immunology, 2005. **175**(11): p. 7135-7142.

14. Davidson, T.S. and E.M. Shevach, *Polyclonal Treg cells modulate T effector cell trafficking.* Eur J Immunol, 2011. **41**(10): p. 2862-70.

15. Chattopadhyay, G. and E.M. Shevach, *Antigen-specific induced T regulatory cells impair dendritic cell function via an IL-10/MARCH1-dependent mechanism.* J Immunol, 2013. **191**(12): p. 5875-84.

16. Huter, E.N., et al., *Cutting Edge: Antigen-Specific TGF -Induced Regulatory T Cells Suppress Th17-Mediated Autoimmune Disease.* The Journal of Immunology, 2008. **181**(12): p. 8209-8213.

17. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.

18. Ng, W.F., et al., *Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells.* Blood, 2001. **98**(9): p. 2736-44.

19. Baecher-Allan, C., et al., *CD4+CD25high regulatory cells in human peripheral blood.* J Immunol, 2001. **167**(3): p. 1245-53.

20. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.

21. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.

22. Roncador, G., et al., *Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level.* Eur J Immunol, 2005. **35**(6): p. 1681-91.

23. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes.* Immunology, 1970. **18**(5): p. 723-37.

24. Vuddamalay, Y. and J.P. van Meerwijk, *CD28- and CD28lowCD8+ Regulatory T Cells: Of Mice and Men.* Front Immunol, 2017. **8**: p. 31.

25. Joosten, S.A., et al., *Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4.* Proc Natl Acad Sci U S A, 2007. **104**(19): p. 8029-34.

26. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse.* Nat Genet, 2001. **27**(1): p. 68-73.

27. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.* Nat Genet, 2001. **27**(1): p. 20-1.

28. Mailer, R.K., K. Falk, and O. Rotzschke, *Absence of leucine zipper in the natural FOXP3Delta2Delta7 isoform does not affect dimerization but abrogates suppressive capacity.* PLoS One, 2009. **4**(7): p. e6104.

29. Allan, S.E., et al., *The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs.* J Clin Invest, 2005. **115**(11): p. 3276-84.

30. Kaur, G., et al., *Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells--identification of Foxp3Delta7 in human regulatory T cells.* Mol Immunol, 2010. **48**(1-3): p. 321-32.

31. Joly, A.L., et al., *Foxp3 lacking exons 2 and 7 is unable to confer suppressive ability to regulatory T cells in vivo.* J Autoimmun, 2015. **63**: p. 23-30.

32. Sadlon, T.J., et al., *Genome-wide identification of human FOXP3 target genes in natural regulatory T cells.* J Immunol, 2 010. **185**(2): p. 1071-81.

33. Birzele, F., et al., *Next-generation insights into regulatory T cells: expression profiling and FoxP3 occupancy in Human.* Nucleic Acids Res, 2011. **39**(18): p. 7946-60.

34. Rudra, D., et al., *Transcription factor Foxp3 and its protein partners form a complex regulatory network.* Nat Immunol, 2012. **13**(10): p. 1010-9.

35. Fu, W., et al., *A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells.* Nat Immunol, 2012. **13**(10): p. 972-80.

36. Lozano, T., N. Casares, and J.J. Lasarte, *Searching for the Achilles Heel of FOXP3.* Front Oncol, 2013. **3**: p. 294.

37. Lee, S.M., B. Gao, and D. Fang, *FoxP3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1.* Blood, 2008. **111**(7): p. 3599-606.

38. Loizou, L., K.G. Andersen, and A.G. Betz, *Foxp3 Interacts with c-Rel to Mediate NF-κB Repression.* PLOS ONE, 2011. **6**(4): p. e18670.

39. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.

40. Wu, Y., et al., *FOXP3 controls regulatory T cell function through cooperation with NFAT.* Cell, 2006. **126**(2): p. 375-87.

41. Lopes, J.E., et al., *Analysis of FOXP3 Reveals Multiple Domains Required for Its Function as a Transcriptional Repressor.* The Journal of Immunology, 2006. **177**(5): p. 3133-3142.

42. Magg, T., et al., *Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells.* Eur J Immunol, 2012. **42**(6): p. 1627-38.

43. Hancock, W.W. and E. Ozkaynak, *Three distinct domains contribute to nuclear transport of murine Foxp3.* PLoS One, 2009. **4**(11): p. e7890.

44. R.Walker, M., et al., *Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25-Tcells.* J. Clin. Invest., 2003. **112**: p. 1437-1443.

45. Aarts-Riemens, T., et al., *Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells.* Eur J Immunol, 2008. **38**(5): p. 1381-90.

46. Moran, A.E. and K.A. Hogquist, *T-cell receptor affinity in thymic development.* Immunology, 2012. **135**(4): p. 261-7.

47. Turner, M.S., L.P. Kane, and P.A. Morel, *Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion.* J Immunol, 2009. **183**(8): p. 4895-903.

48. Molinero, L.L., et al., *High TCR stimuli prevent induced regulatory T cell differentiation in a NF-kappaB-dependent manner.* J Immunol, 2011. **186**(8): p. 4609-17.

49. Plesa, G., et al., *TCR affinity and specificity requirements for human regulatory T-cell function.* Blood, 2012. **119**(15): p. 3420-30.

50. Long, S.A., et al., *Low-dose antigen promotes induction of FOXP3 in human CD4+ T cells.* J Immunol, 2011. **187**(7): p. 3511-20.

51. Sauer, S., et al., *T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7797-802.

52. Sharma, M.D., et al., *The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment.* Sci Adv, 2015. **1**(10): p. e1500845.

53. Tang, Q., et al., *Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells.* J Immunol, 2003. **171**(7): p. 3348-52.

54. Horwitz, D.A., S.G. Zheng, and J.D. Gray, *The role of the combination of IL-2 and TGF-beta or IL-10 in the generation and function of CD4+ CD25+ and CD8+ regulatory T cell subsets.* J Leukoc Biol, 2003. **74**(4): p. 471-8.

55. Chen, Q., et al., *IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo.* J Immunol, 2011. **186**(11): p. 6329-37.

56. Huehn, J., J.K. Polansky, and A. Hamann, *Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage?* Nat Rev Immunol, 2009. **9**(2): p. 83-9.

57. Tone, Y., et al., *Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer.* Nat Immunol, 2008. **9**(2): p. 194-202.

58. Sakaguchi, S., et al., *FOXP3+ regulatory T cells in the human immune system.* Nat Rev Immunol, 2010. **10**(7): p. 490-500.

59. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nat Rev Immunol, 2008. **8**(7): p. 523-32.

60. Patterson, S.J., et al., *T regulatory cell chemokine production mediates pathogenic T cell attraction and suppression.* J Clin Invest, 2016. **126**(3): p. 1039-51.

61. Zheng, S.G., et al., *Generation Ex Vivo of TGF- -Producing Regulatory T Cells from CD4+CD25- Precursors.* The Journal of Immunology, 2002. **169**(8): p. 4183-4189.

62. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. **194**(5): p. 629-44.

63. Andersson, J., et al., *CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner.* J Exp Med, 2008. **205**(9): p. 1975-81.

64. Ito, T., et al., *Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery.* Immunity, 2008. **28**(6): p. 870-80.

65. Hussain, K., et al., *Helicobacter pylori-Mediated Protection from Allergy Is Associated with IL-10-Secreting Peripheral Blood Regulatory T Cells.* Front Immunol, 2016. **7**: p. 71.

66. Collison, L.W., et al., *Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner.* J Immunol, 2009. **182**(10): p. 6121-8.

67. Rubtsov, Y.P., et al., *Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces.* Immunity, 2008. **28**(4): p. 546-58.

68. Collison, L.W., et al., *IL-35-mediated induction of a potent regulatory T cell population.* Nat Immunol, 2010. **11**(12): p. 1093-101.

69. Collison, L.W., et al., *The inhibitory cytokine IL-35 contributes to regulatory T-cell function.* Nature, 2007. **450**(7169): p. 566-9.

70. Choi, B.D., et al., *Human regulatory T cells kill tumor cells through granzyme-dependent cytotoxicity upon retargeting with a bispecific antibody.* Cancer Immunol Res, 2013. **1**(3): p. 163.

71. Gondek, D.C., et al., *Cutting Edge: Contact-Mediated Suppression by CD4+CD25+ Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism.* The Journal of Immunology, 2005. **174**(4): p. 1783-1786.

72. Garin, M.I., et al., *Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells.* Blood, 2007. **109**(5): p. 2058-65.

73. Ocklenburg, F., et al., *UBD, a downstream element of FOXP3, allows the identification of LGALS3, a new marker of human regulatory T cells.* Lab Invest, 2006. **86**(7): p. 724-37.

74. Pfoertner, S., et al., *Signatures of human regulatory T cells: an encounter with old friends and new players.* Genome Biol, 2006. **7**(7): p. R54.

75. Wang, F., et al., *Tim-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells.* Immunobiology, 2009. **214**(5): p. 342-9.

76. Ju, Y., et al., *The Tim-3/galectin-9 pathway involves in the homeostasis of hepatic Tregs in a mouse model of concanavalin A-induced hepatitis.* Mol Immunol, 2014. **58**(1): p. 85-91.

77. Kubach, J., et al., *Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function.* Blood, 2007. **110**(5): p. 1550-8.

78. Mahic, M., et al., *FOXP3+CD4+CD25+ Adaptive Regulatory T Cells Express Cyclooxygenase-2 and Suppress Effector T Cells by a Prostaglandin E2-Dependent Mechanism.* The Journal of Immunology, 2006. **177**(1): p. 246-254.

79. Mandapathil, M., et al., *Adenosine and prostaglandin E2 cooperate in the suppression of immune responses mediated by adaptive regulatory T cells.* J Biol Chem, 2010. **285**(36): p. 27571-80.

80. Borsellino, G., et al., *Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression.* Blood, 2007. **110**(4): p. 1225-32.

81. Mandapathil, M., et al., *Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression.* J Immunol Methods, 2009. **346**(1-2): p. 55-63.

82. Deaglio, S., et al., *Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression.* J Exp Med, 2007. **204**(6): p. 1257-65.

83. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression.* J Exp Med, 2007. **204**(6): p. 1303-10.

84. Fassbender, M., et al., *Cyclic adenosine monophosphate and IL-10 coordinately contribute to nTreg cell-mediated suppression of dendritic cell activation.* Cell Immunol, 2010. **265**(2): p. 91-6.

85. Fallarino, F., et al., *Modulation of tryptophan catabolism by regulatory T cells.* Nat Immunol, 2003. **4**(12): p. 1206-12.

86. Feunou, P., et al., *Foxp3+CD25+ T Regulatory Cells Stimulate IFN- - Independent CD152-Mediated Activation of Tryptophan Catabolism That Provides Dendritic Cells with Immune Regulatory Activity in Mice Unresponsive to Staphylococcal Enterotoxin B.* The Journal of Immunology, 2007. **179**(2): p. 910-917.

87. Cederbom, L., H. Hall, and F. Ivars, *CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells.* Eur J Immunol, 2000. **30**(6): p. 1538-43.

88. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.

89. Park, H.J., et al., *PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells.* J Immunol, 2015. **194**(12): p. 5801-11.

90. Tran, D.Q., et al., *Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function.* J Immunol, 2009. **182**(5): p. 2929-38.

91. Wang, H., et al., *TGF-beta-dependent suppressive function of Tregs requires wild-type levels of CD18 in a mouse model of psoriasis.* J Clin Invest, 2008. **118**(7): p. 2629-39.

92. Wohler, J., et al., *LFA-1 is critical for regulatory T cell homeostasis and function.* Mol Immunol, 2009. **46**(11-12): p. 2424-8.

93. Liang, B., et al., *Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II.* The Journal of Immunology, 2008. **180**(9): p. 5916-5926.

94. Sarris, M., et al., *Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition.* Immunity, 2008. **28**(3): p. 402-13.

95. Bruder, D., et al., *Neuropilin-1: a surface marker of regulatory T cells.* Eur J Immunol, 2004. **34**(3): p. 623-30.

96. Milpied, P., et al., *Neuropilin-1 is not a marker of human Foxp3+ Treg.* Eur J Immunol, 2009. **39**(6): p. 1466-71.

97. Baatar, D., et al., *Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL.* J Immunol, 2007. **178**(8): p. 4891-900.

98. Strauss, L., C. Bergmann, and T.L. Whiteside, *Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis.* J Immunol, 2009. **182**(3): p. 1469-80.

99. Gorbachev, A.V. and R.L. Fairchild, *CD4+CD25+ regulatory T cells utilize FasL as a mechanism to restrict DC priming functions in cutaneous immune responses.* Eur J Immunol, 2010. **40**(7): p. 2006-15.

100. Ren, X., et al., *Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells.* Cell Death Differ, 2007. **14**(12): p. 2076-84.

101. Gilliet, M. and Y.J. Liu, *Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells.* J Exp Med, 2002. **195**(6): p. 695-704.

102. Wei, S., et al., *Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma.* Cancer Res, 2005. **65**(12): p. 5020-6.

103. Filaci, G., et al., *Nonantigen specific CD8+ T suppressor lymphocytes originate from CD8+CD28- T cells and inhibit both T-cell proliferation and CTL function.* Hum Immunol, 2004. **65**(2): p. 142-56.

104. Endharti, A.T., et al., *Cutting Edge: CD8+CD122+ Regulatory T Cells Produce IL-10 to Suppress IFN- Production and Proliferation of CD8+ T Cells.* The Journal of Immunology, 2005. **175**(11): p. 7093-7097.

105. Saitoh, O., et al., *CD8+CD122+ T cells, a newly identified regulatory T subset, negatively regulate Graves' hyperthyroidism in a murine model.* Endocrinology, 2007. **148**(12): p. 6040-6.

106. Rifa'i, M., et al., *Essential roles of CD8+CD122+ regulatory T cells in the maintenance of T cell homeostasis.* J Exp Med, 2004. **200**(9): p. 1123-34.

107. Cosmi, L., et al., *Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes.* Blood, 2003. **102**(12): p. 4107-14.

108. Menager-Marcq, I., et al., *CD8+CD28- regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice.* Gastroenterology, 2006. **131**(6): p. 1775-85.

109. Kang, H.K., et al., *Very Low-Dose Tolerance with Nucleosomal Peptides Controls Lupus and Induces Potent Regulatory T Cell Subsets.* The Journal of Immunology, 2005. **174**(6): p. 3247-3255.

110. Ho, J., et al., *A CD8+/CD103high T Cell Subset Regulates TNF-Mediated Chronic Murine Ileitis.* The Journal of Immunology, 2008. **180**(4): p. 2573-2580.

111. Filaci, G., et al., *Impairment of CD8+ T Suppressor Cell Function in Patients with Active Systemic Lupus Erythematosus.* The Journal of Immunology, 2001. **166**(10): p. 6452-6457.

112. Balashov, K.E., et al., *Inhibition of T cell responses by activated human CD8+ T cells is mediated by interferon-gamma and is defective in chronic progressive multiple sclerosis.* J Clin Invest, 1995. **95**(6): p. 2711-9.

113. Tyler, A.F., et al., *CD8(+) T Cells Are Required For Glatiramer Acetate Therapy in Autoimmune Demyelinating Disease.* PLoS One, 2013. **8**(6): p. e66772.

114. Seo, S.K., et al., *4-1BB-mediated immunotherapy of rheumatoid arthritis.* Nat Med, 2004. **10**(10): p. 1088-94.

115. Wen, Z., et al., *NADPH oxidase deficiency underlies dysfunction of aged CD8+ Tregs.* J Clin Invest, 2016. **126**(5): p. 1953-67.

116. Jarvis, L.B., et al., *Autoreactive human peripheral blood CD8+ T cells with a regulatory phenotype and function.* Eur J Immunol, 2005. **35**(10): p. 2896-908.

117. Chang, C.C., et al., *Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4.* Nat Immunol, 2002. **3**(3): p. 237-43.

118. Bisikirska, B., et al., *TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs.* J Clin Invest, 2005. **115**(10): p. 2904-13.

119. Taylor, A.L., E.L. Cross, and M.J. Llewelyn, *Induction of contact-dependent CD8(+) regulatory T cells through stimulation with staphylococcal and streptococcal superantigens.* Immunology, 2012. **135**(2): p. 158-67.

120. Davila, E., et al., *Cell-Based Immunotherapy with Suppressor CD8+ T Cells in Rheumatoid Arthritis.* The Journal of Immunology, 2005. **174**(11): p. 7292-7301.

121. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.

122. Wang, J., et al., *Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells.* Eur J Immunol, 2007. **37**(1): p. 129-38.

123. Tran, D.Q., H. Ramsey, and E.M. Shevach, *Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype.* Blood, 2007. **110**(8): p. 2983-90.

124. Smith, E.L., et al., *Splice variants of human FOXP3 are functional inhibitors of human CD4+ T-cell activation.* Immunology, 2006. **119**(2): p. 203-11.

125. Bardel, E., et al., *Human CD4+CD25+Foxp3+ Regulatory T Cells Do Not Constitutively Express IL-35.* The Journal of Immunology, 2008. **181**(10): p. 6898-6905.

126. Glinka, Y. and G.J. Prud'homme, *Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity.* J Leukoc Biol, 2008. **84**(1): p. 302-10.

127. Belkaid, Y. and B.T. Rouse, *Natural regulatory T cells in infectious disease.* Nat Immunol, 2005. **6**(4): p. 353-60.

128. Hesse, M., et al., *The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells.* J Immunol, 2004. **172**(5): p. 3157-66.

129. Cabrera, R., et al., *An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection.* Hepatology, 2004. **40**(5): p. 1062-71.

130. Lundgren, A., et al., *Helicobacterpylori-Specific CD4+ CD25high Regulatory T Cells Suppress Memory T-Cell Responses to H. pylori in Infected Individuals.* Infection and Immunity, 2003. **71**(4): p. 1755-1762.

131. Enarsson, K., et al., *Function and recruitment of mucosal regulatory T cells in human chronic Helicobacter pylori infection and gastric adenocarcinoma.* Clin Immunol, 2006. **121**(3): p. 358-68.

132. Guyot-Revol, V., et al., *Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis.* Am J Respir Crit Care Med, 2006. **173**(7): p. 803-10.

133. Zhang, Q., et al., *Characterisation of Regulatory T Cells in Nasal Associated Lymphoid Tissue in Children: Relationships with Pneumococcal Colonization.* PLOS Pathogens, 2011. **7**(8): p. e1002175.

134. Sun, J., et al., *Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10.* Nat Med, 2009. **15**(3): p. 277-84.

135. Trandem, K., et al., *Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis.* J Immunol, 2011. **186**(6): p. 3642-52.

136. MacDonald, A.J., et al., *CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons.* J Infect Dis, 2002. **185**(6): p. 720-7.

137. Garba, M.L., et al., *HIV antigens can induce TGF-beta(1)-producing immunoregulatory CD8+ T cells.* J Immunol, 2002. **168**(5): p. 2247-54.

138. Angin, M., et al., *Acute HIV-1 infection is associated with increased plasma levels of heme oxygenase-1 and presence of heme oxygenase-1-specific regulatory T cells.* Aids, 2017. **31**(5): p. 635-641.

139. Taylor, J.J., M. Mohrs, and E.J. Pearce, *Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population.* J Immunol, 2006. **176**(10): p. 5839-47.

140. Doetze, A., et al., *Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift.* Int Immunol, 2000. **12**(5): p. 623-30.

141. Kang, S.A., et al., *Parasitic nematode-induced CD4+Foxp3+T cells can ameliorate allergic airway inflammation.* PLoS Negl Trop Dis, 2014. **8**(12): p. e3410.

142. Montagnoli, C., et al., *Immunity and tolerance to Aspergillus involve functionally distinct regulatory T cells and tryptophan catabolism.* J Immunol, 2006. **176**(3): p. 1712-23.

143. Cavassani, K.A., et al., *Systemic and local characterization of regulatory T cells in a chronic fungal infection in humans.* J Immunol, 2006. **177**(9): p. 5811-8.

144. Wilson, G.J., et al., *A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia.* PLoS Pathog, 2011. **7**(10): p. e1002271.

145. Ono, H.K., et al., *Identification and Characterization of a Novel Staphylococcal Emetic Toxin.* Appl Environ Microbiol, 2015. **81**(20): p. 7034-40.

146. Llewelyn, M. and J. Cohen, *Superantigens: microbial agents that corrupt immunity.* The Lancet Infectious Diseases, 2002. **2**(3): p. 156-162.

147. Thomas, D., et al., *Staphylococcus aureus superantigens elicit redundant and extensive human Vbeta patterns.* Infect Immun, 2009. **77**(5): p. 2043-50.

148. Seo, K.S., et al., *A quantitative real time PCR method to analyze T cell receptor Vbeta subgroup expansion by staphylococcal superantigens.* J Transl Med, 2010. **8**: p. 2.

149. Arad, G., et al., *Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation.* Nat Med, 2000. **6**(4): p. 414-21.

150. Brosnahan, A.J., et al., *Novel toxic shock syndrome toxin-1 amino acids required for biological activity.* Biochemistry, 2008. **47**(49): p. 12995-3003.

151. Arad, G., et al., *Binding of superantigen toxins into the CD28 homodimer interface is essential for induction of cytokine genes that mediate lethal shock.* PLoS Biol, 2011. **9**(9): p. e1001149.

152. Krakauer, T., *Immune response to staphylococcal superantigens.* Immunol Res, 1999. **20**(2): p. 163-73.

153. Rammensee, H.G., R. Kroschewski, and B. Frangoulis, *Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells.* Nature, 1989. **339**(6225): p. 541-4.

154. Huang, L. and I.N. Crispe, *Superantigen-driven peripheral deletion of T cells. Apoptosis occurs in cells that have lost the alpha/beta T cell receptor.* J Immunol, 1993. **151**(4): p. 1844-51.

155. Wang, Z.Q., et al., *Staphylococcal enterotoxin B-induced T-cell anergy is mediated by regulatory T cells.* Immunology, 1998. **94**(3): p. 331-9.

156. Jiang, H., et al., *Murine CD8+ T cells that specifically delete autologous CD4+ T cells expressing V beta 8 TCR: a role of the Qa-1 molecule.* Immunity, 1995. **2**(2): p. 185-94.

157. Feunou, P., et al., *CD4+CD25+ and CD4+CD25- T Cells Act Respectively as Inducer and Effector T Suppressor Cells in Superantigen-Induced Tolerance.* The Journal of Immunology, 2003. **171**(7): p. 3475-3484.

158. Miller, C., J.A. Ragheb, and R.H. Schwartz, *Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms in vivo.* J Exp Med, 1999. **190**(1): p. 53-64.

159. Lee, J., et al., *Induction of Immunosuppressive CD8(+)CD25(+)FOXP3(+) Regulatory T Cells by Suboptimal Stimulation with Staphylococcal Enterotoxin C1.* J Immunol, 2018. **200**(2): p. 669-680.

160. Klein, E., D.L. Smith, and R. Laxminarayan, *Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005.* Emerg Infect Dis, 2007. **13**(12): p. 1840-6.

161. Hafer, C., et al., *Contribution of selected gene mutations to resistance in clinical isolates of vancomycin-intermediate Staphylococcus aureus.* Antimicrob Agents Chemother, 2012. **56**(11): p. 5845-51.

162. Gillet, Y., et al., *Factors predicting mortality in necrotizing community-acquired pneumonia caused by Staphylococcus aureus containing Panton-Valentine leukocidin.* Clin Infect Dis, 2007. **45**(3): p. 315-21.

163. Lavoie, P.M., et al., *Understanding the mechanism of action of bacterial superantigens from a decade of research.* Immunol Rev, 1999. **168**: p. 257-69.

164. Kappler, J., et al., *V beta-specific stimulation of human T cells by staphylococcal toxins.* Science, 1989. **244**(4906): p. 811-3.

165. McCormick, J.K., J.M. Yarwood, and P.M. Schlievert, *Toxic shock syndrome and bacterial superantigens: an update.* Annu Rev Microbiol, 2001. **55**: p. 77-104.

166. White, J., et al., *The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice.* Cell, 1989. **56**(1): p. 27-35.

167. Migita, K. and A. Ochi, *The fate of anergic T cells in vivo.* J Immunol, 1993. **150**(3): p. 763-70.

168. Stach, C.S., et al., *Novel Tissue Level Effects of the Staphylococcus aureus Enterotoxin Gene Cluster Are Essential for Infective Endocarditis.* PLoS One, 2016. **11**(4): p. e0154762.

169. Kuroishi, T., et al., *Inflammatory responses of bovine polymorphonuclear neutrophils induced by staphylococcal enterotoxin C via stimulation of mononuclear cells.* Clin Diagn Lab Immunol, 2003. **10**(6): p. 1011-8.

170. Seo, K.S., et al., *Long-term staphylococcal enterotoxin C1 exposure induces soluble factor-mediated immunosuppression by bovine CD4+ and CD8+ T cells.* Infect Immun, 2007. **75**(1): p. 260-9.

171. Tollersrud, T., A.H. Kampen, and K. Kenny, *Staphylococcus aureus enterotoxin D is secreted in milk and stimulates specific antibody responses in cows in the course of experimental intramammary infection.* Infect Immun, 2006. **74**(6): p. 3507-12.

172. Josefowicz, S.Z., L.F. Lu, and A.Y. Rudensky, *Regulatory T cells: mechanisms of differentiation and function.* Annu Rev Immunol, 2012. **30**: p. 531-64.

173. Belkaid, Y., *Regulatory T cells and infection: a dangerous necessity.* Nat Rev Immunol, 2007. **7**(11): p. 875-88.

174. Maizels, R.M. and K.A. Smith, *Regulatory T cells in infection.* Adv Immunol, 2011. **112**: p. 73-136.

175. Apostolou, I. and H. von Boehmer, *In vivo instruction of suppressor commitment in naive T cells.* J Exp Med, 2004. **199**(10): p. 1401-8.

176. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen.* Nat Immunol, 2005. **6**(12): p. 1219-27.

177. Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.

178. Ciubotariu, R., et al., *Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+CD28- regulatory T cells.* J Immunol, 1998. **161**(10): p. 5193-202.

179. Zheng, Y., et al., *Acquisition of suppressive function by activated human CD4+ CD25- T cells is associated with the expression of CTLA-4 not FoxP3.* J Immunol, 2008. **181**(3): p. 1683-91.

180. Blomster-Hautamaa, D.A. and P.M. Schlievert, *Preparation of toxic shock syndrome toxin-1.* Methods Enzymol, 1988. **165**: p. 37-43.

181. Fehervari, Z. and S. Sakaguchi, *Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells.* Int Immunol, 2004. **16**(12): p. 1769-80.

182. Snodgrass, R.G., et al., *Mitomycin C inhibits ribosomal RNA: a novel cytotoxic mechanism for bioreductive drugs.* J Biol Chem, 2010. **285**(25): p. 19068-75.

183. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

184. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.

185. Ernst, P.B., J.C. Garrison, and L.F. Thompson, *Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology.* J Immunol, 2010. **185**(4): p. 1993-8.

186. Suzuki, M., et al., *CD8+CD45RA+CCR7+FOXP3+ T cells with immunosuppressive properties: a novel subset of inducible human regulatory T cells.* J Immunol, 2012. **189**(5): p. 2118-30.

187. Trapani, J.A., *Granzymes: a family of lymphocyte granule serine proteases.* Genome Biol, 2001. **2**(12): p. Reviews3014.

188. Kappler, J.W., et al., *A T cell receptor V beta segment that imparts reactivity to a class II major histocompatibility complex product.* Cell, 1987. **49**(2): p. 263-71.

189. Strauss, L., et al., *Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin.* J Immunol, 2007. **178**(1): p. 320-9.

190. Basu, S., et al., *Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin.* J Immunol, 2008. **180**(9): p. 5794-8.

191. Singh, K., et al., *Regulatory T cells exhibit decreased proliferation but enhanced suppression after pulsing with sirolimus.* Am J Transplant, 2012. **12**(6): p. 1441-57.

192. Kaempfer, R., et al., *CD28: direct and critical receptor for superantigen toxins.* Toxins (Basel), 2013. **5**(9): p. 1531-42.

193. Sarantopoulos, S., L. Lu, and H. Cantor, *Qa-1 restriction of CD8+ suppressor T cells.* J Clin Invest, 2004. **114**(9): p. 1218-21.

194. Tennakoon, D.K., et al., *Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis.* J Immunol, 2006. **176**(11): p. 7119-29.

195. Perillo, N.L., et al., *Apoptosis of T cells mediated by galectin-1.* Nature, 1995. **378**(6558): p. 736-9.

196. Cedeno-Laurent, F. and C.J. Dimitroff, *Galectin-1 research in T cell immunity: past, present and future.* Clin Immunol, 2012. **142**(2): p. 107-16.

197. Gondek, D.C., et al., *Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.* J Immunol, 2005. **174**(4): p. 1783-6.

198. Becker, K., et al., *Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO, and SElN.* Mol Nutr Food Res, 2004. **48**(7): p. 488-95.

199. Fueyo, J.M., et al., *Relationships between toxin gene content and genetic background in nasal carried isolates of Staphylococcus aureus from Asturias, Spain.* FEMS Microbiol Lett, 2005. **243**(2): p. 447-54.

200. Moon, B.Y., et al., *Phage-mediated horizontal transfer of a Staphylococcus aureus virulence-associated genomic island.* Sci Rep, 2015. **5**: p. 9784.

201. Moon, B.Y., et al., *Mobilization of Genomic Islands of Staphylococcus aureus by Temperate Bacteriophage.* PLoS One, 2016. **11**(3): p. e0151409.

202. Schlievert, P.M., et al., *Secreted virulence factor comparison between methicillin-resistant and methicillin-sensitive Staphylococcus aureus, and its relevance to atopic dermatitis.* J Allergy Clin Immunol, 2010. **125**(1): p. 39-49.

203. Oderup, C., et al., *Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression.* Immunology, 2006. **118**(2): p. 240-9.

204. Crellin, N.K., R.V. Garcia, and M.K. Levings, *Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells.* Blood, 2007. **109**(5): p. 2014-22.

205. Miskov-Zivanov, N., et al., *The duration of T cell stimulation is a critical determinant of cell fate and plasticity.* Sci Signal, 2013. **6**(300): p. ra97.

206. Elola, M.T., et al., *Galectin-1 receptors in different cell types.* J Biomed Sci, 2005. **12**(1): p. 13-29.

207. He, J. and L.G. Baum, *Presentation of galectin-1 by extracellular matrix triggers T cell death.* J Biol Chem, 2004. **279**(6): p. 4705-12.

208. Kovacs-Solyom, F., et al., *Mechanism of tumor cell-induced T-cell apoptosis mediated by galectin-1.* Immunol Lett, 2010. **127**(2): p. 108-18.

209. Madireddi, S., et al., *Regulatory T Cell-Mediated Suppression of Inflammation Induced by DR3 Signaling Is Dependent on Galectin-9.* J Immunol, 2017. **199**(8): p. 2721-2728.

210. Delgoffe, G.M., et al., *The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment.* Immunity, 2009. **30**(6): p. 832-44.

211. Haxhinasto, S., D. Mathis, and C. Benoist, *The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells.* J Exp Med, 2008. **205**(3): p. 565-74.

212. Waickman, A.T. and J.D. Powell, *mTOR, metabolism, and the regulation of T-cell differentiation and function.* Immunol Rev, 2012. **249**(1): p. 43-58.

213. Zeng, H., et al., *mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function.* Nature, 2013. **499**(7459): p. 485-90.

214. Hawse, W.F., et al., *Cutting Edge: Differential Regulation of PTEN by TCR, Akt, and FoxO1 Controls CD4+ T Cell Fate Decisions.* J Immunol, 2015. **194**(10): p. 4615-9.

215. Lavoie, P.M., et al., *Understanding the mechanism of action of bacterial supernatigens from a decade of research.* Immunol Rev, 1999. **168**: p. 257-269.

216. Suzuki, M., et al., *Inhibitory CD8+ T cells in autoimmune disease.* Hum Immunol, 2008. **69**(11): p. 781-9.

217. Song, X., et al., *Structural and biological features of FOXP3 dimerization relevant to regulatory T cell function.* Cell Rep, 2012. **1**(6): p. 665-75.

218. Bandukwala, H.S., et al., *Structure of a domain-swapped FOXP3 dimer on DNA and its function in regulatory T cells.* Immunity, 2011. **34**(4): p. 479-91.

219. Pillai, V., et al., *Transient regulatory T-cells: a state attained by all activated human T-cells.* Clin Immunol, 2007. **123**(1): p. 18-29.

220. Huijts, C.M., et al., *mTOR Inhibition Per Se Induces Nuclear Localization of FOXP3 and Conversion of Invariant NKT (iNKT) Cells into Immunosuppressive Regulatory iNKT Cells.* J Immunol, 2015. **195**(5): p. 2038-45.

221. Chen, C., et al., *Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation.* J Biol Chem, 2006. **281**(48): p. 36828-34.

222. Desmet, F.O., et al., *Human Splicing Finder: an online bioinformatics tool to predict splicing signals.* Nucleic Acids Res, 2009. **37**(9): p. e67.

223. Ip, J.Y., et al., *Global analysis of alternative splicing during T-cell activation.* RNA, 2007. **13**(4): p. 563-72.

224. Martinez, N.M., et al., *Alternative splicing networks regulated by signaling in human T cells.* RNA, 2012. **18**(5): p. 1029-40.

225. Hawse, W.F., W.C. Boggess, and P.A. Morel, *TCR Signal Strength Regulates Akt Substrate Specificity To Induce Alternate Murine Th and T Regulatory Cell Differentiation Programs.* J Immunol, 2017. **199**(2): p. 589-597.

226. Chen, L., et al., *mTORC2-PKBalpha/Akt1 Serine 473 phosphorylation axis is essential for regulation of FOXP3 Stability by chemokine CCL3 in psoriasis.* J Invest Dermatol, 2013. **133**(2): p. 418-28.

227. Beier, U.H., et al., *Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms.* Sci Signal, 2012. **5**(229): p. ra45.

228. van Loosdregt, J., et al., *Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization.* Blood, 2010. **115**(5): p. 965-74.

229. Carbone, F., et al., *Regulatory T cell proliferative potential is impaired in human autoimmune disease.* Nat Med, 2014. **20**(1): p. 69-74.

230. De Rosa, V., et al., *Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants.* Nat Immunol, 2015. **16**(11): p. 1174-84.

231. Lundberg, A.K., et al., *Activation-induced FOXP3 isoform profile in peripheral CD4+ T cells is associated with coronary artery disease.* Atherosclerosis, 2017. **267**: p. 27-33.

232. Serena, G., et al., *Proinflammatory cytokine interferon-gamma and microbiome-derived metabolites dictate epigenetic switch between forkhead box protein 3 isoforms in coeliac disease.* Clin Exp Immunol, 2017. **187**(3): p. 490-506.

233. Fu, S., et al., *TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors.* Am J Transplant, 2004. **4**(10): p. 1614-27.

234. Notredame, C., D.G. Higgins, and J. Heringa, *T-Coffee: A novel method for fast and accurate multiple sequence alignment.* J Mol Biol, 2000. **302**(1): p. 205-17.

APPENDIX A

SUPPLIMENTARY FIGURES

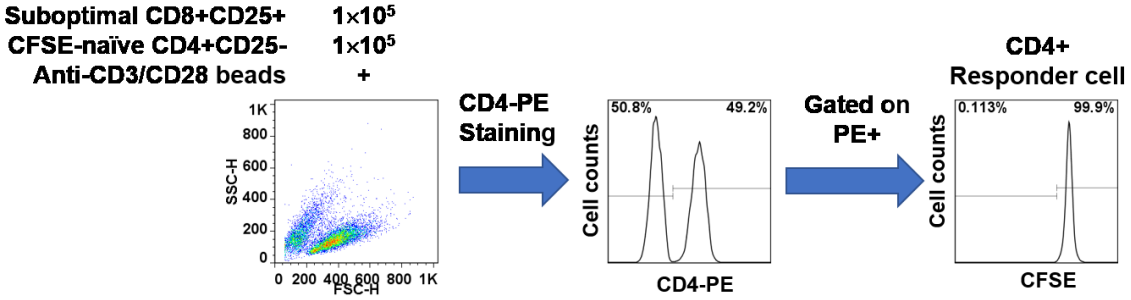

Figure A.1 Gating strategy to separate CD4+CD25- responder T cells from CD8+CD25+or- T cells in autologous MLR.

After co-culturing CFSE-labeled CD4+CD25- T cells with CD8+CD25+ T cells induced from stimulation with SEC1 in autologous MLR, cells were stained with an anti-CD4-PE mAb. In flow cytometry analysis, cells positive for a PE signal (CD4+ responder cells) were gated to separate CD8+CD25+ T cells (PE signal negative). The dilution of CFSE signal is detected to measure proliferation of CD4+ responder cells.

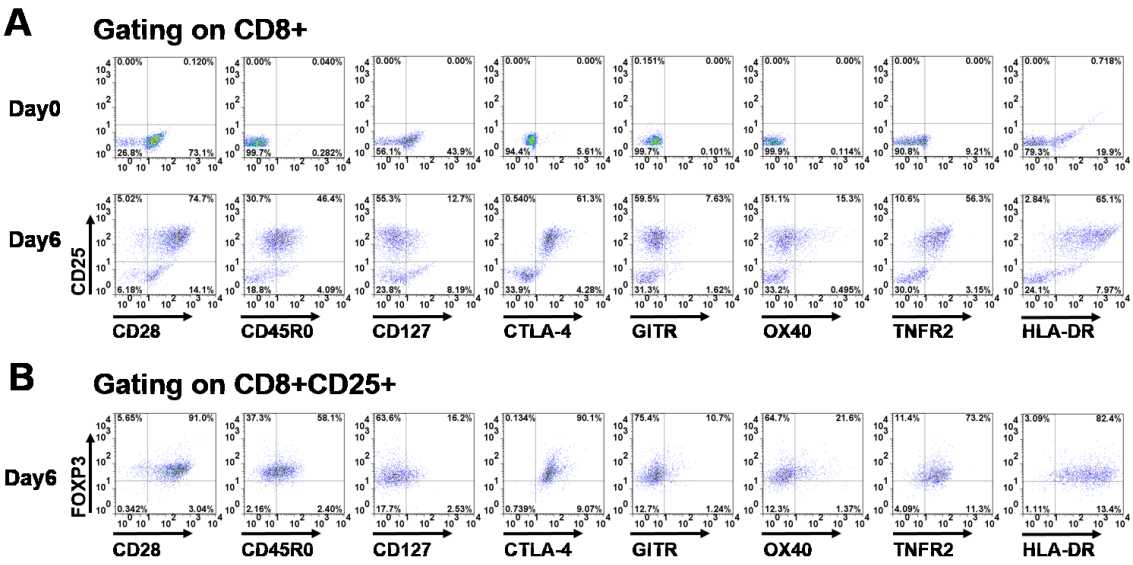

Figure A.2 Phenotypic characterization of CD8+CD25+FOXP3+ T cells induced from suboptimal stimulation with SEC1.

(A) Human PBMCs depleted of CD25+ T cells were stimulated with a suboptimal stimulation concentration of SEC1 (1 ng/ml) for 6 days. Expression of costimulatory molecules related to regulatory T cells were measured by flow cytometry. Data shown are gated on CD8+ T cells. (B) Data shown are gated on CD8+CD25+ T cells to analyze expression of markers related to regulatory T cells in relation with FOXP3. Data shown are representative results from nine independent experiments measured from three different donors.

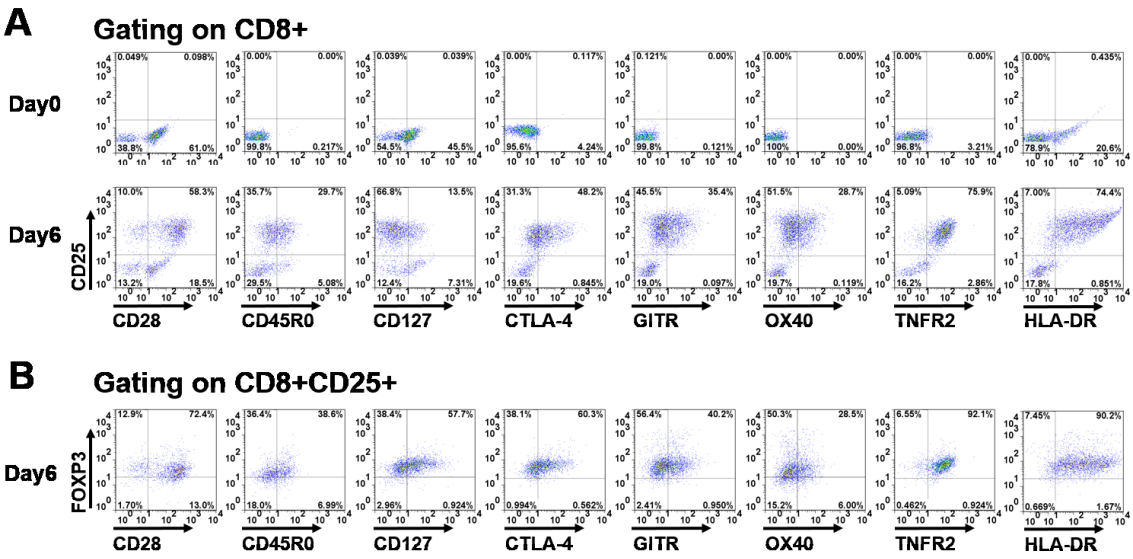

Figure A.3 Phenotypic characterization of CD8+CD25+FOXP3+ T cells induced from optimal stimulation with SEC1.

(A) Human PBMCs depleted of CD25+ T cells were stimulated with an optimal stimulation concentration of SEC1 (1 μg/ml) for 4 days. Expression of costimulatory molecules related to regulatory T cells were measured by flow cytometry. Data shown are gated on CD8+ T cells. (B) Data shown are gated on CD8+CD25+ T cells to analyze expression of markers related to regulatory T cells in relation with FOXP3. Data shown are representative results from nine independent experiments measured from three different donors.

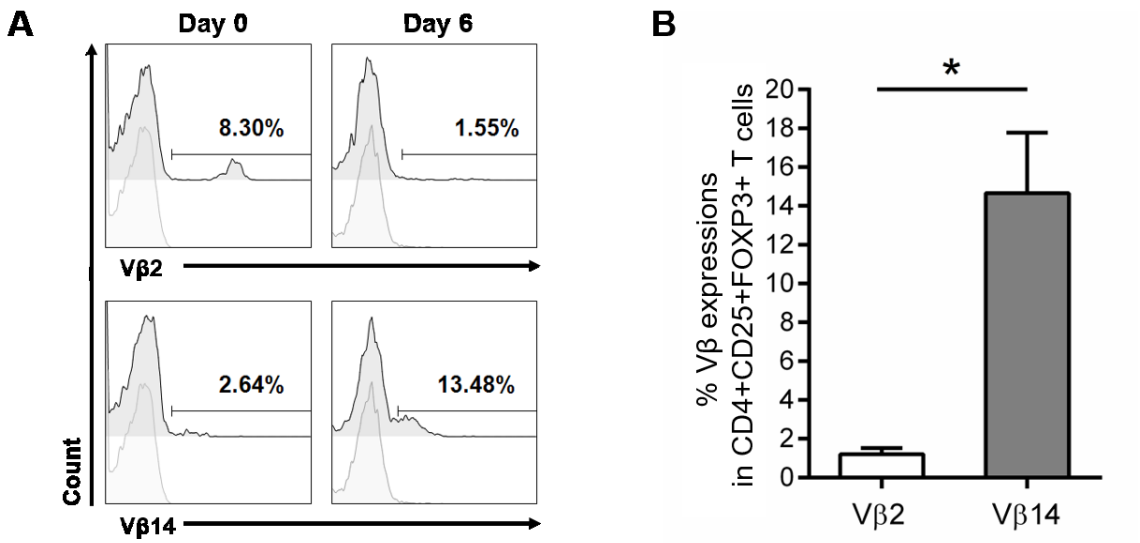

Figure A.4 Vβ-specific induction of CD4+CD25+FOXP3+ T cells by suboptimal stimulation with SEC1.

Expressions of Vβ2 and Vβ14, representing non-specific and specific Vβ subset to SEC1 respectively, were measured by flow cytometry before and after stimulation with SEC1 (1 ng/ml) for 6 days. (A) Percentage of Vβ positive population (dark grey, upper histogram) was measured based on FMO control (light grey, lower histogram) and cells were gated in CD4+. (B) Percentage of Vβ positive population was measured in CD4+CD25+FOXP3+ cells after suboptimal stimulation for 6 days.

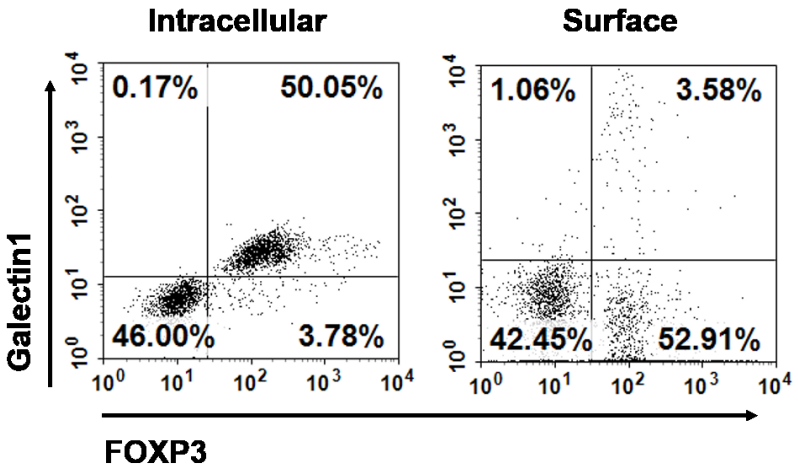

Figure A.5 Galectin-1 expression in CD4+CD25+ T cells from optimal stimulation.

Human PBMCs depleted of CD25+ T cells were stimulated with 1 µg/ml of SEC1 for 4 days and galectin-1 expression was measured after intracellular and surface staining using flow cytometry. Data shown are gated on CD4+CD25+ T cells.

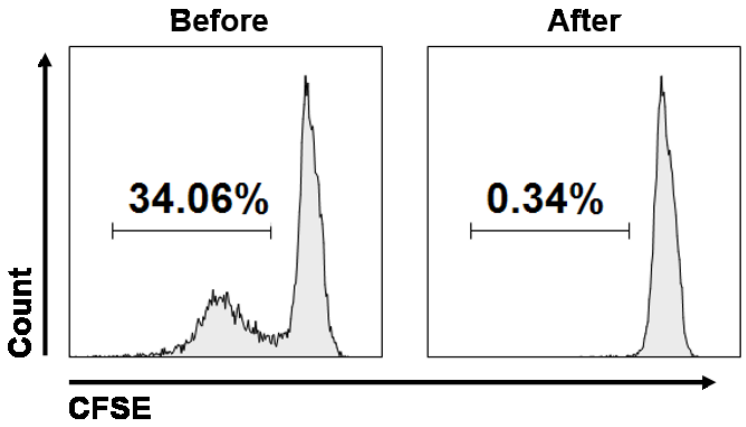

Figure A.6 The remaining SEC1 in conditioned media is completely removed.

Conditioned media was collected from culture supernatant of PBMCs stimulated with 1 ng/ml of SEC1 (suboptimal stimulation) for 6 days. Collected conditioned media was flown through resin column and remaining his-tagged SEC1 was removed by nickel-affinity binding. Human PBMCs were isolated, stained with CFSE, and cultured in conditioned media before and after SEC1 removal. Proliferation of cells was measured on day 4 by flow cytometry.

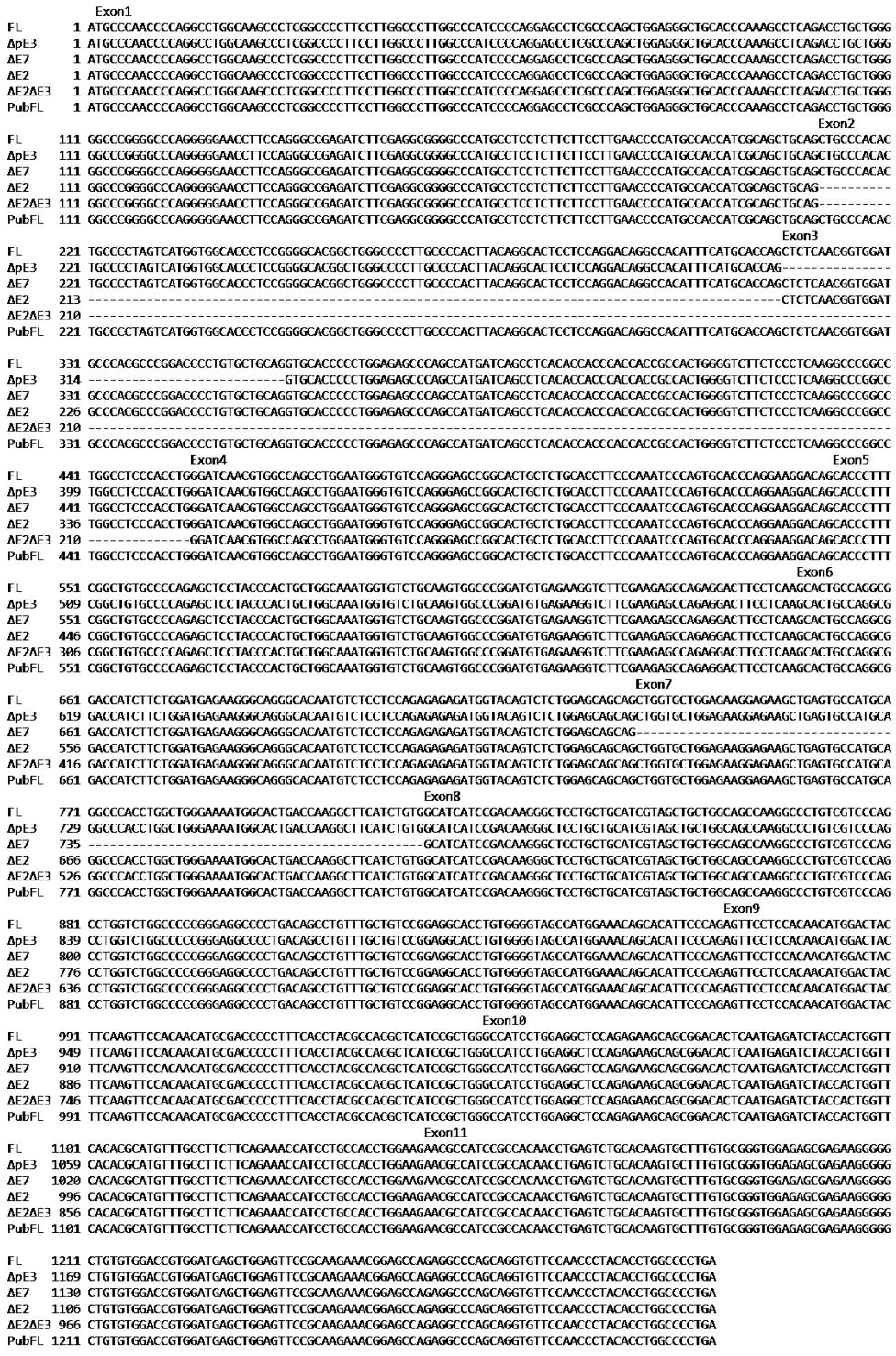

Figure A.7 Multiple sequence alignment of FOXP3 splice variants.

Nucleotide sequences identified by DNA sequencing was aligned using T-coffee [234]. FL: FOXP3 full-length, PubFL: published nucleotide sequence of full-length FOXP3.